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SECTION MEETINGS

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Biochemical Properties of Sulfonamide-resistant Strains of *Streptococcus mitis,* and Their Similarity to Enterococci. (17609)

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In a previous investigation(1) of the properties of sulfathiazole-resistant green streptococci, it was found that 4 strains which were originally unable to grow on 40% bile agar, acquired this ability after a high level of sulfonamide resistance had been induced. Sherman, Niven, and Smiley(2), Evans and Chinn(3), and Swift(4) have indicated that the ability to grow on bile agar, in .1% methylene blue milk, in 6.5% NaCl broth, and under certain other conditions not usually considered as optimum for the streptococci, is useful in differentiating the enterococci from the viridans group. It seemed of interest to determine whether any other properties characteristic of the enterococci (in addition to the growth on 40% bile) were acquired by these strains of *Streptococcus mitis* in becoming sulfathiazole-resistant. Further, since enterococci are resistant to penicillin and streptomycin, it was of interest to determine the resistance of the "trained" *mitis* strains to these antibiotics, and to compare the resistance of these trained strains and of enterococci to bacitracin* and aureomycin. Finally, it seemed desirable to develop bile resistance and streptomycin resistance in the parent *Streptococcus mitis* cultures and to determine whether these

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1. Clapper, William E., and Heatherman, Mary E., Proc. Soc. Exp. Biol. and Med., 1948, v68, 392.

2. Sherman, J. M., Niven, C. F., and Smiley, K. L., *J. Bact.*, 1943, v45, 249.

3. Evans, Alice C., and Chinn, Alice L., *J. Bact.*, 1947, v54, 495.

4. Swift, Homer F., *The Streptococci: Bacterial and Mycotic Infection in Man,* J. B. Lippincott Co., Phila., Pa., p. 253, 1948.

* We are indebted to the Lederle Laboratories for kindly supplying the aureomycin and to the Upjohn Company for the bacitracin.

TABLE I.
Biochemical Properties of Sulfathiazole-resistant Streptococci.

	Parent strains				Resistant strains			
	1	4	6	17	1-S	4-S	6-S	17-S
Mg sulfa/ml*	.1	.1	.1	.1	>30	>30	>30	20
Growth in								
40% bile	—	—	—	—	+	+	+	+
6.5% NaCl	—	—	—	—	+	+	+	+
10°C	—	—	—	—	+	+	+	—
45°C	—	+	—	—	+	+	+	+
.1% M. B. Milk	—	—	—	—	+	—	+	+
Reduction, before clotting, of Litmus Milk	—	—	—	—	+	—	+	—

* Concentration inhibiting growth in broth.

agents would select organisms with the same properties, or bring about the same changes, as those found after exposure to sulfathiazole.

Methods and Materials. Two of the strains of *Streptococcus mitis* from our previous study(1) which had been isolated from the mouth, and two additional strains, one isolated from the mouth and one from the urine, were streaked on blood agar and re-isolated from single colonies 8 times. They were then made resistant by growing in gradually increasing concentrations of sodium sulfathiazole in tryptose phosphate broth. The parent strains were also trained in the same manner to grow on 40% bile agar and in high concentrations of streptomycin. Several unsuccessful attempts were made to induce penicillin resistance. The sensitivity tests to sulfathiazole and the antibiotics were performed by making dilutions (usually 5-fold) of the various agents in tryptose phosphate broth, inoculating with the organism, and reading the lowest concentration completely preventing growth after 24 hours at 37°C, to determine the sensitivity of the parent strain and reading the highest concentration allowing good growth to determine the resistance of the resistant strain. Inoculum size was equalized by adjusting the 24-hour broth cultures of the parent and resistant strains to the same turbidity, by visual inspection, before diluting 1 to 1,000. One drop of this dilution was then added to each tube.

Results and discussion. The results shown in Table I indicate that after a high degree of resistance to sulfathiazole had been attained, organisms that originally had the properties of *Streptococcus mitis* now had certain properties of enterococci. Table II compares the sensitivity to antibiotics of the sulfathiazole-resistant strains to that of the susceptible ones. Although strains of bacteria trained to sulfonamide resistance are usually as sensitive to the antibiotics as the parent strains, the ones in this study were more resistant. A known strain of *Streptococcus fecalis* (A.T.C.C. No. 9790) showed very much the same sensitivity to the 4 antibiotics as did the sulfonamide-resistant organisms. All the trained organisms and the *S. fecalis* were sensitive to less than 1 μg/ml of aureomycin or bacitracin.

Table III shows the properties acquired by the parent strains when they were trained to grow on 40% bile agar. Strain 4B acquired all the properties of the enterococci that were shown by the sulfonamide-resistant cells including increase in sulfathiazole resistance. Strain 17B did not become sulfathiazole-resistant, nor acquire the other properties of an enterococcus, but did become more resistant to the 4 antibiotics. The remaining 2 strains showed no changes. When the parent strains were made 1,000 times or more resistant to streptomycin they did not change in resistance to any of the other agents, and showed no change in other biochemical properties, with the exception that 3 strains acquired the ability to grow on 40% bile agar. These results emphasize the fact that development of resistance to one bacteriostatic agent does not necessarily bring about the same metabolic

TABLE II.
Resistance of Parent and Sulfathiazole-resistant Strains of Streptococci to Antibiotics.

Strain	Aureomycin	Bacitracin	Penicillin	Streptomycin
Parent 1	.05*	.01	1.0‡	5
Resistant 1	.5†	.05	10.	35
Parent 4	.05	.01	.5	5
Resistant 4	.5	.05	10.	25
Parent 6	.1	.01	1.0	5
Resistant 6	.5	.05	10.	25
Parent 17	.005	.001	.5	5
Resistant 17	.5	.01	10.	25

* Values represent concentration in μg/ml required to prevent growth for 24 hr.

† Values represent concentration in μg/ml through which good growth of the organism was obtained in 24 hr.

‡ Values for penicillin are given in units per ml.

TABLE III.
Biochemical Reactions and Resistance of Streptococci Trained to Grow on 40% Bile Agar.

	Strains trained on bile			
	1-B	4-B	6-B	17-B
Growth on				
40% Bile	+	+	+	+
10°C	—	—	—	—
45°C	—	+	—	—
.1% M. B. Milk	—	+	—	—
Reduction, before clotting, of Litmus Milk	—	+	—	+
Increase in sulfathiazole resistance	—	+	—	—
Increase in resistance to aureomycin, bacitracin, penicillin, and streptomycin	—	10-fold or more to all 4	—	10-fold or more to all 4

change as development of resistance to another. Each agent produces specific metabolic changes(5).

Enterococci are resistant to sulfathiazole, penicillin, streptomycin, and bile. If organisms having all these properties were originally present in small numbers in close association with the parent type, any of these agents would select organisms having the properties of enterococci. Furthermore, since our resistant organisms grow much faster than the parent strains, they would grow out in broth with no sulfathiazole in it, if they were already present. However, the same number of transfers were made in drug-free broth as were made in the presence of sulfathiazole, and no change in resistance was observed. To test further the possibility that a few variants possessing the properties of our resistant strains were present in the culture before exposure to sulfathiazole the following experiment was performed. Approximately 4 resistant cells of strain 6S, checked by plate counts of dilution, were mixed with 10^9 susceptible cells of strain 6 in tryptose phosphate broth. After 24 hours incubation, the sulfathiazole resistance of this mixed culture was determined. It was found to be the same as that of a pure culture of the resistant strain (6S). This would seem to be fairly good evidence that the parent culture did not have even a few highly resistant cells in it. Since the parent culture did not produce a resistant culture when transferred in drug-free broth, variants having the properties of enterococci must have been induced or selected stepwise by the sulfathiazole. Such a change might take place in a patient treated with sulfonamides, with the resulting organisms being resistant to penicillin and streptomycin and having other prop-

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erties of an enterococcus. Such an organism would apparently be inhibited by low concentrations of bacitracin or aureomycin, however. The results of our *in vitro* experiments suggest that resistance developed in *mitis* streptococci by contact with other agents does not necessarily bring about the overall increase in resistance shown to accompany sulfonamide resistance.

Summary. 1. At a certain level of sulfonamide resistance properties characteristic of enterococci were acquired by 4 strains of *S. mitis*. 2. Increased resistance to sulfonamides was accompanied by increased resistance to penicillin, streptomycin, bacitracin, and aureomycin in all 4 strains. The resistance of these trained strains to the antibiotics and sulfathiazole was similar to that of a strain of *S. fecalis*. 3. Acquisition of ability to grow on bile or in high concentrations of streptomycin did not generally produce the biochemical properties of enterococci, nor resistance to sulfathiazole or to the antibiotics.

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Influence of Certain Neurotropic Substances on Central and Synaptic Transmission in Callianassa. (17610)

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Although acetylcholine and cholinesterase have been shown to be present in the nervous system of crustaceans, evidence for the functional significance of these substances is generally lacking(1,2,3,4). A possible exception may occur in the anomuran *Petrolisthes armatus,* in which Welsh and Haskin(5) have found that injection of acetylcholine increases the tendency to autotomy of the walking legs. In this animal eserine in doses of 1.5 γ per gram elicited extreme activity and convulsions, while atropine exerted a depressive action. No attempt was made by Welsh and Haskin to localise the site of action of these substances. In an effort to obtain further information on the action of these and other neurotropic agents, we have carried out experiments on the macrurous anomuran *Callianassa californiensis* in which application of reagents was made at 3 different loci: 1. On the abdominal nerve cord and ganglia. 2. At the point of synapse between giant fibers of the nerve cord and the nerves to the flexors of the telson. 3. On the flexor muscles of the abdomen after severance of their connections with the central nervous system.

Methods. The abdominal nerve cord of *Callianassa* usually was exposed from the ventral side, freed entirely from adjoining tissues and left continuous with the thoracic cord. The abdomen of the animal was then cut away and the head and thorax, with the attached nerve cord, placed in a bath of sea water. Preparations kept well in running sea water for periods up to 24 hours. The drugs were made up in sea water and added in the proper concentrations directly to the (40 cc) bath. Electrical stimulation and recording were carried out by means of micromanipulated platinum electrodes. Responses were amplified through a Grass P3 amplifier and visualised on the screen of a DuMont 208 oscilloscope, the sweep of which was triggered from the stimulating circuit. The flexor muscles of the abdomen were denervated by exposing and removing the entire abdominal nerve cord from the dorsal side, leaving the muscles and their attachments intact. The synapses between the nerves to the flexors of the telson and the giant fibers of the cord (there are two giant fibers in this form, one on each side of the abdominal cord) made a favorable preparation for the study of possible synaptic effects. Unless otherwise stated, stimuli were delivered at approximately 30 per minute.

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* Special Fellow, National Institute of Health, 1948-49.

† Department of Biology, University of Portland, Ore.

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Observations. Eserine. Intact animals in which eserine (physostigmine salicylate) was allowed access to the ventral nerve cord displayed vigorous and sustained muscular responses. On the isolated nerve cord eserine (2.5×10^{-3} M) after a latent period of 15 to 30 seconds caused marked bursts of activity (Fig. 1). Activity of the ventral nerve cord could also be brought about by weaker solutions (2.5×10^{-5} M) after a latent period of 3 to 3.5 minutes. In concentrations of 2.5×10^{-4} M the reaction was prompt, 15 to 30 seconds, and the amplitude of the response was maximal, since increase of the concentration to 2.5×10^{-3} M produced no further increase in amplitude. This "spontaneous" activity continued undiminished for an hour. At concentrations as high as 2.5×10^{-3} M eserine had no effect on the conduction characteristics of the giant fibers, nor on transmission through the giant fiber-telson nerve synapse. Denervated muscle likewise showed no response to eserine in these concentrations.

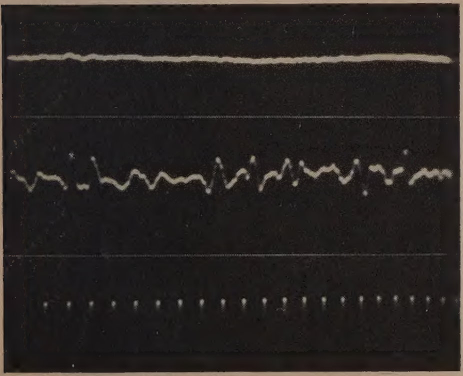

FIG. 1.
Top: resting baseline. Middle: activity of ventral nerve cord produced by 2.5×10^{-3} M eserine. Bottom: Time line, 1,000 cycles per sec.

Acetylcholine (1×10^{-2} M) alone did not affect the reactivity of the cord, the synapses or denervated muscle. When applied within a few seconds after eserine there was no clear augmentation of response, such as is characteristic in indisputably cholinergic forms.

Atropine (2.5×10^{-3} M) after a latent period of approximately 10 minutes brought about a significant increase in "spontaneous" activity of the abdominal cord. This activity often reached an intensity comparable to that seen with eserine, and was maintained for 30 minutes or longer. No effects were observed on synaptic transmission nor on conduction in the giant fibers, nor did any depression phase appear over a period of 90 minutes.

Nicotine was found to block synaptic transmission reversibly in concentrations in the neighborhood of 1×10^{-5} M to 4×10^{-5} M. Conduction in the giant fibers was unaffected by nicotine in these concentrations.

Methylamine hydrochloride blocked synaptic transmission in concentrations of 1×10^{-3} M. Washing with sea water effected complete recovery of synaptic function.

Triethylamine hydrochloride in concentrations of 1×10^{-3} M produced no observable effects, but 4×10^{-3} M completely blocked synaptic transmission. Function could be effectively restored by simple washing with sea water.

Di-isopropyl fluorophosphate (DFP) at concentrations of 1.3×10^{-2} M immediately blocked both conduction and synaptic transmission for a few seconds, after which these functions spontaneously recovered and remained normal for an experimental period of 60 minutes. Concentrations of 2.8×10^{-2} M blocked synaptic transmission for 20 minutes and conduction in the giant fibers for approximately 10 minutes. At concentrations above 3.0×10^{-3} M spontaneous activity was noticeable and persisted even after 10-12 hours washing in sea water.

Discussion. While in general the reactions of *Callianassa* to the reagents tested followed the pattern of other crustacea (Table I), there are certain points of difference, especially in the responses to eserine and atropine. Eserine in concentrations of 1×10^{-3} pro-

TABLE I.

Drug	No. Exp.	Block conduction in giant fibers	Block synapses of giant fibers	Increase "spontaneous" activity in cord
Eserine	15	—	—	++++
Acetylcholine	12	—	—	—
Atropine	5	—	—	+++
Nicotine	5	—	+	—
DFP	3	+ (transient)	+ (transient)	+
Triethylamine	3	—	+	—
Methylamine	3	—	+	—

duces increased activity in the nerve cord of the crayfish (Prosser(6)); whereas solutions only 1% as strong are effective in exciting the nerve cord of *Callianassa.* Thus the neurons of *Callianassa* are responsive to eserine in amounts similar to those to be expected in a normal physiological system. This central excitatory action of eserine most probably underlies the responses observed in *Petrolisthes* by Welsh and Haskin(5). Prosser(6) is inclined to attribute the effects of eserine to a direct toxic action on the nerve cells. It would appear that atropine also exerts such an action, at least in the concentrations (2.5×10^{-3} M) employed by us. (Prosser(6) noted no effect of 10^{-4} atropine in the crayfish.) The depressive action of atropine observed in *Petrolisthes* by Welsh and Haskin(5) may have been peripheral in origin, since an effect of atropine similar to that of novocaine and procaine has been observed in nerve-muscle preparations of the crayfish by Ellis, Thienes and Wiersma(7).

The actions of nicotine, methylamine, triethylamine and DFP on *Callianassa* are in essential accord with the data obtained on the crayfish by Wiersma and Schallek(8).

It is to be noted that, although eserine exerts an excitatory action on the neurons of the abdominal cord of *Callianassa* in concentrations low enough to be physiologically significant, the addition of acetylcholine to an eserinised preparation has no observable further effect. This is true even when the eserine is causing "submaximal" excitation, or if acetylcholine is added during the latent period preceding marked activation of the neurons. Thus, even though it might be possible to attribute the excitatory effects of eserine and DFP to their anticholinesterasic activity(9), the failure of acetylcholine itself to produce any characteristic effect leads us to conclude that a cholinergic mechanism does not play a predominant role in the excitation and conduction phenomena of *Callianassa*. The action of eserine is probably therefore referable to its properties as a central excitant.

Summary. Eserine (2.5×10^{-5} M to 2.5×10^{-3} M) and atropine (2.5×10^{-3} M) produce a marked increase in the "spontaneous" activity of the ganglion cells of the ventral nerve cord of *Callianassa californiensis*. These substances have no appreciable effect on conduction characteristics of the giant fibers in the nerve cord, on the transmission across the synapse between the giant fibers and the nerves to the flexors of the telson, nor on denervated muscle. Nicotine (4×10^{-5} M), methylamine (1×10^{-3} M), triethylamine (4×10^{-3} M) and di-isopropyl fluorophosphate (DFP) (1.3×10^{-2} M) reversibly block synaptic transmission for varying periods of time.

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Relationship of Vitamin B_6 to the Metabolism of D-Amino Acids.* (17611)

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In the course of work upon D-amino acid oxidase (the enzyme which deaminates D-amino acids to their corresponding alpha-keto acids) it appeared possible that vitamin B_6 might influence the activity of this enzyme(1). This vitamin is known to be the coenzyme for transaminase enzymes, presumably acting as carrier for the amino group through the formation of a Schiff's base between pyridoxal phosphate and the amino acid(2,3,4). The transaminase reaction has been conclusively demonstrated only with L-amino acids. A similar mechanism appeared possible in the oxidative deamination of D-amino acids. This hypothesis was tested by assaying liver and kidney tissues from vitamin B_6 deficient and control rats for D-amino acid oxidase activity. Although preliminary experiments showed that the activity of the enzyme in deficient animals was lower than that of control animals fed *ad libitum*, pair feeding of the animals reduced the D-amino acid oxidase activity of the control almost to that of the depleted animals.

The recent work of Holden, Furman and Snell(5) has shown that vitamin B_6 can be substituted for D-alanine in the growth of *L. casei* and *S. faecalis*, and that this vitamin promotes the synthesis of D-alanine by these organisms. It was suggested that vitamin B_6 acts as a coenzyme in the synthesis of D-alanine. Their work stimulated a reinvestigation of the effect of vitamin B_6 on the utilization of certain D-amino acids by the rat. It has been demonstrated that the net nitrogen utilization of vitamin B_6 deficient animals which have been fed supplements of D-amino acids is only 30% of that of animals receiving simultaneous injection of pyridoxine. The reaction appears fairly specific, in that the utilization of nitrogen by B_6 deficient animals fed L-amino acids or nitrogen in the inorganic form is not affected to a similar extent.

Experimental. Male albino rats weighing 40-50 g were obtained at weaning from Sprague-Dawley and placed in individual wire cages. The basal ration employed for the production of a vitamin B_6 deficiency was that of Sarma, Snell, and Elvehjem(6), consisting of sucrose 73 g, blood fibrin 18 g, corn oil 5 g, salts IV 4 g, thiamin 0.2 mg, riboflavin 0.3 mg, nicotinic acid 2.5 mg, calcium pantothenate 2 mg, choline chloride 100 mg, 2-methyl, 1,4-naphthoquinone 1 mg, inositol, 10 mg, biotin 0.01 mg, and folic acid .3 mg per 100 g of diet. Two drops of haliver oil were administered weekly. Control animals received the same ration with 0.25 mg % of pyridoxine·HCl added. Water was supplied *ad libitum*. One group of control animals used for enzyme studies was fed *ad libitum*, *all* other control groups were pair-fed with the deficient animals. Growth of pair-fed controls closely paralleled that of the deficient animals. A B_6 deficiency was manifested by an almost immediate and continued excretion of xanthurenic acid by animals on the deficient ration.

D-amino acid oxidase activity of rat tissues was determined by the method of Axelrod, Sober and Elvehjem(7), which measures oxy-

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TABLE I.
D Amino Acid Oxidase Activity of Vitamin B_6-Deficient and Control Rat Tissues.

		Series I—Controls fed *ad libitum* Q_{O_2}		Series II—Pair-fed controls Q_{O_2}	
Group		Liver	Kidney	Liver	Kidney
B_6-deficient	Avg	1.76 ± .29 (5)	8.24 ± .98 (4)	3.07 ± .69 (6)	12.3 ± 2.90 (6)
Control	Avg	2.42 ± .75 (6)	25.1 ± 6.82 (5)	3.05 ± .72 (5)	15.7 ± 6.22 (5)

Period on experimental rations varied from one to 12 weeks. Enzyme activity determined as described in text. Variation expressed as standard deviation from the mean. Figures in parentheses represent the number of determinations.

gen consumption with DL-alanine as substrate. Each Warburg flask contained 1.5 cc of Krebs-Ringer phosphate buffer, pH 7.4, 1 cc of tissue homogenate equivalent to 200 mg of fresh liver or 100 mg of fresh kidney tissue, 0.5 cc of 0.3 M DL-alanine, and 0.2 cc of 5 N NaOH in the center well. *In vitro* additions of pyridoxine, pyridoxamine, pyridoxal, and pyridoxal phosphate were made at a level of 10-50 μg per flask. Flasks were incubated at 37°C, readings of oxygen uptake were made at ten minute intervals for a period of one hour. All solutions and homogenates were made in glass redistilled water. Endogenous uptake in the absence of alanine was determined in each case and values deducted from those with added substrate. All data shown are averages from duplicate flasks which consistently checked closely. Oxygen uptake per hour was calculated from the two highest values for 10 minute periods. Activity is expressed as QO_2 values, which indicate μl O_2/hour/mg dry weight of tissue.

For the metabolism studies, all animals were maintained on the above rations for 4 weeks; at this time their weights ranged from 56 to 77 g in the deficient and 69 to 83 g in the control lots. The animals were then transferred to "low protein" rations, identical with those above except that the blood fibrin was reduced to 6% to the advantage of the sucrose, and fed these rations until the conclusion of the experiment.

After one week on the low protein rations, animals were placed in individual wire metabolism cages equipped with glass funnels. Urine was collected in bottles containing sulfuric acid and toluene; feces were collected separately on wire screens. Collections were made daily. Nitrogen in both excrements was determined by the Kjeldahl method. Several days were allowed the animals for adjustment to new conditions before the start of the experiment; all animals were in positive nitrogen balance when supplemented.

Animals receiving B_6 were injected intraperitoneally with 200 μg of pyridoxine·HCl in aqueous solution twice within an interval of 18 hours. Seven hours after the final injections of B_6, supplements of D-amino acids, L-amino acids, or di-ammonium citrate were given to animals as indicated in Table II. Amino acid supplements were composed of molecular equivalent weights of the crystalline L or D isomers of leucine, isoleucine, phenylalanine, and valine in aqueous solution, adjusted to pH 7.2, and administered by stomach tube to supply exactly 24 mg of nitrogen per animal. Di-ammonium citrate solutions were administered similarly to supply 24 mg of nitrogen per animal. Gavage supplements totalling 4-6 cc of the solution were given in two doses within a 24 hour period. Nitrogen excretion was followed until levels had reached the basal level established before supplementation. In each case this was reached by the third day; data are therefore given for the two days following supplementation.

Results. Data for D-amino acid oxidase determinations are given in Table I. Initial observations indicated that D-amino acid oxidase activity of the B_6 deficient animals was considerably lower than that of the control animals (Series I). When the experiment was repeated using pair-fed controls, there was no significant difference between the deficient and control groups (Series II). The effect of *in vitro* additions of pyridoxine, pyridoxamine, pyridoxal, or pyridoxal phosphate,

TABLE II.

Effect of Vitamin B_6 on the Utilization of Amino Acids and Inorganic Nitrogen.

Supplement	B_6 deficient animals				Control animals			
	*Total N ingested mg	N excreted mg	% N utilized	Avg % utilized	*Total N ingested mg	N excreted mg	% N utilized	Avg % utilized
I None	126.8	53.3	58	56 ± 2.6	140.9	50.2	66	66
	122.8	58.6	52					
	112.3	48.1	57					
II B_6 (400 μg)	151.7	67.2	56	65 ± 7.8	138.1	43.2	68	68
	205.5	51.3	75					
	154.3	55.7	64					
III D-amino acids	92.1	80.2	12	21 ± 9.0	162.2	60.0	63	54 ± 6.8
	131.6	83.0	37		210.0	91.8	56	
	122.8	87.8	29		139.2	78.3	44	
	161.4	128.1	21		163.2	74.5	54	
	108.4	95.7	12					
	151.1	124.6	18					
IV D-amino acids + B_6 (400 μg)	144.0	66.5	54	60 ± 5.5	165.0	56.7	66	66
	219.9	76.4	65					
V L-amino acids	203.0	99.0	51	39 ± 10.5	177.6	92.2	48	47 ± 9.9
	116.2	80.2	32		159.2	104.8	34	
	138.2	74.0	46		147.0	78.8	46	
	122.9	92.6	25†		163.0	68.5	58	
VI L-amino acids + B_6 (400 μg)	198.8	95.6	52	52	176.6	98.3	44	44
VII $(NH_4)_2$ citrate	139.2	91.2	36	33 ± 4.9	152.6	88.2	42	47 ± 10.3
	119.1	79.9	33		152.6	96.0	37	
	138.2	103.8	25†		167.9	64.8	61	

* Summation of dietary N and N from supplement. All animals received supplements as indicated to supply 24 mg N/animal. Values represent totals of two days following supplementation; each series of values represents a separate animal. Variation expressed as standard deviation from the mean.

† Severe deficiencies resulting from a longer period on the depletion diet may have affected total nitrogen utilization in these animals.

with and without ATP, upon the activity of tissues from B_6 deficient animals was tested frequently, and consistently failed to result in any increase in D-amino acid oxidase activity. The determinations were repeated on a third series of animals at a later time with the same results. The function of certain vitamins as coenzymes for enzyme systems has been demonstrated frequently by a lowered enzyme activity in animals deficient in that vitamin. Thus D-amino acid oxidase activity is decreased in rats deficient in riboflavin(7), and a B_6 deficiency has been shown to result in decreased transaminase activity(4). We have been unable to demonstrate a relationship between D-amino acid oxidase and vitamin B_6 by this technic.

Results of nitrogen balance studies upon animals given D-amino acids by gavage supplementation are shown in Table II. The data indicate that utilization of dietary nitrogen by animals deficient in vitamin B_6 is less than that of the control animals. Injection of the deficient animals with pyridoxine raises the nitrogen utilization to the same level as that of the control groups. Upon administration of D-amino acids, the B_6 deficient animal excretes a much geater amount of total nitrogen than does an animal with an adequate supply of the vitamin. Excretion of the excess nitrogen appears to be almost entirely in the urine of the first day's collection; however, nitrogen excretion is not again "normal" until the third day, necessitating use of two day excretion data. Administration of D-amino acids to the B_6 deficient animal depresses the level of nitrogen utilization to about one third of the normal value (Group III). Injection of the animals with pyridoxine prior to administration of the D-amino acids (Group IV) raises the level of nitrogen utilized to that of the untreated controls (Group I).

To determine whether a specific relationship between vitamin B_6 and D-amino acids is involved, the same amounts of nitrogen were administered as the L-isomers of the previously used amino acids or in the inorganic form as di-ammonium citrate. The administration of nitrogen in either of these forms decreases the net nitrogen utilization of the B_6 deficient animal, while that of the control groups is not thus affected. However, the effect is considerably less than that noted upon supplementation with D-amino acids. The net nitrogen utilization of the B_6 deficient animal is lowered to 21% by the administration of D-amino acids, while administration of L-amino acids or di-ammonium citrate decreases utilization only to 39% or 33% respectively. Exceptionally low values for nitrogen utilization by B_6 deficient animals given D-amino acids result from the excretion of a large percentage of dietary nitrogen in addition to that furnished by the D-amino acids. In the case of supplements with L-amino acids or inorganic nitrogen, the difference between deficient and control animals can be accounted for almost entirely by total excretion of the supplementary nitrogen. It has been demonstrated that ammonium nitrogen can be utilized by the rat fed only essential amino acids(8). However, no net utilization of ammonium nitrogen occurs in rats fed complete proteins. The administration of D-amino acids to the B_6 deficient animal appears to result in the excretion of large amounts of dietary nitrogen utilized under ordinary conditions. That these effects are related specifically to a B_6 deficiency and not to the nitrogen supplementation alone is shown by the observation that nitrogen utilization of the control animals is not adversely affected, and that the effect is completely overcome by simultaneous administration of pyridoxine to the B_6 deficient rats.

Discussion. The data demonstrate that the administration of D-amino acids, L-amino acids, or di-ammonium citrate decreases the net nitrogen utilization of the B_6 deficient animal, and that simultaneous injection of pyridoxine completely overcomes this effect. Lowered utilization is most pronounced following the administration of D-amino acids, the utilization of nitrogen after feeding L-amino acids or di-ammonium citrate being affected to a lesser degree. B_6 has been shown to be intimately related to protein metabolism and is known to be involved specifically in

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8. Lardy, H. A., and Feldott, G., *J. Biol. Chem.*, 1949, v179, 509.

the transaminase reaction and amino acid decarboxylation; blocking these reactions by a deficiency of B_6 would be expected to decrease the utilization of L-amino acids and ammonium citrate. A mechanism by which B_6 may affect the utilization of D-amino acids has not been demonstrated. The data indicate that the administration of D-amino acids to the B_6 deficient animal causes the excretion of a large amount of dietary nitrogen which is otherwise utilized. The mechanism by which the toxicity of D-serine, administered by stomach tube, is reduced by simultaneous administration of pyridoxine(9) may be related to this effect. Since the only demonstrated reaction for the metabolism of D-amino acids is through the action of D-amino acid oxidase, it is possible that a deficiency of B_6 decreases the utilization of D-amino acids by its effect upon this enzyme,

Summary. Homogenized kidney from rats deficient in vitamin B_6 exhibited only one third as much D-amino acid oxidase activity as did normal rat kidney homogenates. However, when the food intake of control animals was restricted to that of the B_6 deficient group there was no significant difference between the two groups in their kidney D-amino acid oxidase activity.

A gavage supplement of D-amino acids (leucine, isoleucine, phenylalanine and valine) to vitamin B_6 deficient rats greatly depresses dietary nitrogen utilization. Simultaneous injection of, or an adequate dietary supply of, vitamin B_6 completely overcomes this effect.

Supplements of the L-isomers of the same amino acids or an equivalent amount of inorganic nitrogen effected a smaller depression of dietary nitrogen utilization, thus pointing to a specific relationship between D-amino acid metabolism and vitamin B_6.

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Adrenal Function and Blood Electrolytes. (17612)

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Prevailing concepts attribute special physiological significance to the levels of the blood sodium and potassium in relation to steroid hormone function of the adrenal cortex. They are based on the observation that a reduction of sodium and increase of potassium occurs in the blood serum of adrenalectomized animals, and that administration of desoxycorticosterone or its acetate can effect a reversal of that change. It is maintained that this "corticoid" is a specific hormone of the adrenal cortex whose function is to regulate the balance between those electrolytes in the blood. The probability that loss of something other than any of the adrenal steroids might be responsible for the disturbed electrolyte levels in the blood of adrenalectomized animals has not been given serious attention. In view of the fact that adrenalectomy deprives an animal of function of the adrenal medulla as well as of alleged functions of cortical steroids, we undertook this investigation to determine if the physiological secretion of epinephrine might exercise an influence upon the blood sodium and potassium. A possible relationship between epinephrine action and serum potassium is suggested, also, from the results of various pharmacological investigations.

The demonstration by Rogoff and Stewart(1,2) that sodium chloride is diminished in the blood of completely adrenalectomized dogs included the fact that at least 20% of the animals did not develop this change although other changes were present and the animals succumbed to the results of adrenal cortical insufficiency. Bauman and Kurland(3) obtained similar evidence and added the information that an elevation of plasma potassium occurred in adrenalectomized cats. Others have come to consider this as the pathognomonic evidence of adrenal cortical insufficiency although the idea does not have conclusive and unequivocal experimental support. In many instances the increase in potassium can be regarded as a concomitant of hemoconcentration. Significant evidence on this question is available from the electrical conductivity, specific gravity, and refractometric measurements that were reported by Rogoff and Stewart(1,2). The pharmacodynamic effects of adrenalin include an influence upon serum potassium. It seemed possible that the physiological secretion of epinephrine from the adrenal medulla might be concerned with regulation of potassium in the blood, perhaps more so than the influence of the supposed steroid hormone, desoxycorticosterone. This question lends itself to satisfactory quantitative study. The results of such an investigation are reported here.

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* Supported by the G. N. Stewart Memorial Fund.

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There is agreement on the part of different investigators that administration of adrenalin does not result in a significant change in the level of blood sodium. The dosage employed in various studies upon human subjects as well as experimental laboratory animals was such as to induce hyperglycemia, glycosuria, elevation of blood pressure, and lymphocytosis, with or without a concomitant elevation in the level of blood serum potassium(4,5,6). However, conflicting results have been recorded in the literature concerning the influence of adrenalin upon serum potassium. Our experiments indicate that the discrepancies in the results of different investigators probably can be explained by the differences in dosage and method or rate of administration of the adrenalin. In this connection, is may be pointed out that dosage determines whether injected adrenalin produces only a glycemic response or both hyperglycemia and arterial hypertension. It has been reported that an increase in serum potassium is essential for production of a concomitant rise in blood pressure(7). Such an effect requires a much larger dose of adrenalin than is effective in producing a rise in blood sugar. It was found by McGuigan and Higgins(7) that the potassium ion, in certain dosage, is capable of eliciting responses which simulate sympathomimetic action of epinephrine. This observation, together with the fact that administration of adrenalin was found to increase the level of potassium in the blood serum, led Camp and Higgins(8) to conclude that the effects of adrenalin are, indeed, really due to the action of potassium. Camp(9) remarked that "The increase in serum potassium in Addison's disease would seem to be due to improper distribution of the potassium ion consequent to epinephrine deficiency."

Examination of the results reported by a number of investigators reveals that an elevation of the level of potassium followed administration of adrenalin in those cases where the dosage was relatively high, compared with the known range of physiological secretion of the hormone from the adrenals, or where smaller doses were introduced into the circulation at a rapid rate. In some instances the dosage and rate of injection of the adrenalin were such as yielded a preliminary, transient, rise in potassium which was succeeded by a more sustained decline lasting about one hour and then returning to the

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initial level(10,11,12).

In the observations made by Keys(11) on normal men, intravenous administration of adrenalin resulted in a decline in the level of potassium. In experimental laboratory animals, increase of serum potassium followed the injection of adrenalin. The different results can be accounted for by difference in dosage and rate of administration, *i.e.*, different amounts and concentrations of adrenalin in circulation. Brewer, Larson and Schroeder(12), in similar experiments, reported a preliminary rise followed by a fall in potassium, induced by adrenalin in both man and laboratory animals. Commenting on the difference between their results and those of Keys, they state, "In man as in the dog an interval of several minutes between injection of epinephrine and blood sampling allows sufficient time for the transient rise to have been replaced by a significant decrease in the blood potassium level."

It appears to us that the difference in results probably is due to the fact that in the experiments upon human subjects, by Keys, the effective dosage of adrenalin was in the physiological rather than in the pharmacological range. It was remarked by Brewer, *et al.* that "the possibility that a 'pure' fall in serum potassium might be produced if the proper dosage and injection rate for epinephrine could be found immediately presented itself." Obviously, this was accomplished in the experiments on men, by Keys.

Our experiments illustrate that the proper dosage and injection rate for eliciting a fall in the blood serum potassium is easily determined. If adrenalin is introduced into the blood stream in an amount and at a rate that corresponds to the physiological rate of epinephrine secretion by the adrenals, only a decline in potassium is produced in dogs. Other effects result from administration of doses that are above the physiological range. Such pharmacological reactions may result also from too rapid injection of smaller amounts of adrenalin.

Stewart and Rogoff(13) established that the average physiological rate of epinephrine secretion from the adrenals is between 0.0002 mg and 0.00025 mg per kg of body weight per minute. When administered at such a rate, our experiments indicate that adrenalin always causes a decline in the level of serum potassium. Therefore, we believe that this effect is a physiological reaction. Accordingly, it is highly probable that a quantitative influence upon the regulation of the level of blood potassium is exercised by the physiological epinephrine secretion from the adrenal medulla.

All of our experiments were performed on dogs. Preliminary studies were made to determine the normal range of blood serum sodium and potassium for dogs, under the ordinary conditions of our laboratory. The influence of physiological dosage of adrenalin upon the serum levels of these electrolytes was studied *a* in normal dogs, *b* in dogs with reduction or suppression of epinephrine secretion from the adrenals, *c* completely adrenalectomized animals, with and without administration of interrenalin (adrenal cortex extract).† All of the measurements of sodium and potassium were made by two of us (J.M.Q. and A.W.R.), employing the flame photometer; the operative and experimental procedures were performed by the other two (J.M.R. and E.N.N.). Adrenalin was administered by constant intravenous injection employing the apparatus described by Rogoff(14).

In nearly all of the experiments in which adrenalin was administered a blood specimen was obtained before the injection was begun, another after about 45 minutes of injection and a third about an hour after the injection was discontinued. In some of the experiments a specimen was also taken about 2 to 3 minutes after the beginning of the injection.

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TABLE I.
Effect of Constant Intravenous Injection of Physiological Quantities of Adrenalin upon Serum Sodium and Potassium in Normal Dogs and in Dogs After Reduction or Suppression of the Epinephrine Secretion from the Adrenal Glands. Dog, ♂.

		Adrenalin inj.					
		Before		During		After	
Date	Dose	Na	K	Na	K	Na	K
11/25/47	.14	153.8	5.27	144.6	5.27		
				152.4	4.60	160.9	3.59
28	.28	162.8	4.58	142.3	5.38		
				158.4	4.02	150.7	4.98
12/16	.28	148.9	4.76	145.2	5.04		
					4.47	154.8	4.87
19	.14	152.3	5.96	149.9	6.21		
				154.5	6.23	160.7	5.90
1/23/48				Right adrenal denervated			
26	.28	144.1	5.00	152.2	4.40		
				144.9	5.88	145.0	5.26
29	.14	172.9	5.65	169.1	4.97		
				178.1	5.21	163.7	5.47
2/ 6				Left adrenal excised			
9	.15	148.0	6.27	147.8	5.13		
				149.8	5.47	148.9	5.34
3/ 4	.72	152.1	5.87	161.4	4.17	154.0	5.13
30	.30	161.4	4.14	149.1	4.06	165.6	5.16
4/13				Right adrenal denervated further			
16	.30	109.3	5.36	107.7	5.73	117.3	4.66
20	.30	126.5	4.63	137.8	3.95	129.7	4.51
26	.15	154.8	4.90			150.1	3.72

The doses and rate of injection of adrenalin corresponded to amounts that were within the range which represents the minimum and maximum epinephrine output from the adrenal glands, under ordinary experimental conditions, *i.e.*, between approximately 0.0001 mg, and 0.001 mg per kg of body weight per minute. Seventy-one determinations on blood specimens obtained at intervals from 11 normal dogs yielded an average of 143 milliequivalents per liter for sodium. Sixty-nine determinations on the same animals and mostly on the corresponding blood specimens yielded an average of 5.1 milliequivalents per liter for potassium.

Double adrenalectomy was performed in 7 dogs. Two of these were treated with the adrenal cortical hormone, interrenalin. In 5 dogs, one adrenal was excised and the other denervated, an interval being allowed between operations for a series of blood studies. The remaining (denervated) adrenal was finally excised in one of these dogs, thus adding another to the group of double adrenalectomies. The remaining 4 animals were subjected to repeated denervation of the gland, later, to control the possibility of regeneration of the secretory nerves. These 4 dogs remained alive. Table I illustrates the results obtained in one of the dogs after suppression of epinephrine secretion. Table II presents experiments with 2 dogs, after bilateral adrenalectomy. In the 12 experimental animals, a total of 142 determinations of K and Na in the blood serum were made before the operations, 74 after excision or denervation of one adrenal, 138 after denervation or excision of the second adrenal, and 39 after repeated denervation or excision of the remaining gland. In all, 393 determinations were made in these experimental animals, in addition to the preliminary series that was performed on the normal, control dogs.

In general, the results of our experiments indicate that constant intravenous administration of adrenalin, in physiological dosage, does not influence the level of blood serum sodium. A small elevation that sometimes was observed could be accounted for by the salt added to the circulating blood in the isotonic

TABLE II.
Effect of Constant Intravenous Injection of Physiological Quantities of Adrenalin upon Blood Sodium and Potassium in Completely Adrenalectomized Dogs.

			Adrenalin injection						
			Before		During		After		
Dog	Date	Dose	Na	K	Na	K	Na	K	Remarks
A	6/9/47	.13	112.6	7.52	124.6	5.54	126.0	5.36	2nd adrenal excised 6/6/47. Died a few hrs after adrenalin inj.
B	9	.20	137.0	5.16	133.4	3.70	138.2	3.98	2nd adrenal excised 6/6/47. Cortical extr. 6/7/47 to 7/5/47.
	7/8	.20	120.5	6.31	123.3	4.06	129.5	5.12	Extr. discontinued 3 days.
	11	.20	144.4	7.66	145.7	5.87	145.9	6.11	Extr. discontinued 6 days.

saline solution in which the adrenalin dilutions were prepared. This was of the same order of magnitude as was found when saline solution, without added adrenalin, was administered as a control experiment. On the other hand, diminution in the level of potassium nearly always (about 90% of cases) occurred. The results were the same in normal dogs, in those in which the adrenal epinephrine output was reduced or suppressed, and in bilaterally adrenalectomized animals in which elevation of potassium in the blood serum was present with or without diminution of the sodium level. While there were indications that the relation between epinephrine action and potassium level in the blood might be quantitative, it would require many more experiments to determine that point with certainty.

In completely adrenalectomized dogs, the increase in serum potassium often was associated with a decline in sodium. Frequently, the rise in potassium occurred without a change in the sodium and, occasionally, a fall in sodium level was found in the absence of a significant change in potassium. Alteration in the level of one or both of the electrolytes after adrenalectomy sometimes was found early but more often it developed and became more marked as the animal's condition became critical and onset of a moribund state was evident. In a significant number of experiments, there was a moderate rise in potassium, without change in sodium, for a day or two after unilateral adrenalectomy or adrenal denervation. In cases of bilateral excision of the glands, such a temporary rise in potassium level may occur after each operation. When the second adrenal is excised, however, that rise in potassium usually is followed by a more marked increase developing with the onset of the phenomena of acute adrenal cortical insufficiency. If the adrenal cortical hormone, interrenalin, is administered in proper dosage following excision of the second adrenal, the early temporary rise in potassium sometimes occurs and the more marked change is not seen until after cessation of the hormone treatment, when it develops usually about the same time as after second adrenalectomy in untreated animals.

These observations, together with the fact that the increase in blood serum potassium can be prevented or corrected by administration of epinephrine in such amounts as are liberated normally from the adrenal glands, suggest the possibility or, indeed, the probability that any physiological role of the adrenal gland in the regulation of blood potassium is concerned with function of the epinephrine secretion from the adrenal medulla rather than the alleged function of a specific steroid hormone, actual secretion of which by the cortex has not been proven.

In some of our experimental animals one adrenal was denervated or excised and later the order of operation was reversed on the remaining gland. A third operation was performed, still later, to effect more marked reduction or total suppression of epinephrine secretion from the remaining gland (denervated). In a few experiments this gland was finally excised, placing the animal in the group of dogs with bilateral adrenalectomy. The results of experiments on constant intravenous injection of adrenalin, in amounts that correspond with the physiological epinephrine output from the adrenals, are illustrated in Table I. This experimental animal was selected for the table as a sample because it demonstrates, in one and the same animal, results obtained on the effect of adrenalin under normal conditions, after partial reduction of the animal's epinephrine secretion from the adrenals, and after operation for further reduction or suppression of the adrenal secretion.

The table illustrates that a reduction of blood serum potassium is effected by the action of physiological quantities of adrenalin. Amounts that are equivalent to the minimal rate of epinephrine secretion from the adrenals are effective in normal animals and in those that have been subjected to reduction or suppression of the epinephrine secretion. In completely adrenalectomized animals the same amounts of adrenalin are effective in diminishing the blood serum potassium regardless of whether it has or has not been elevated following loss of the adrenals.

An observation of some interest was made in a few of the experiments. It was found that in cases where there was a high potassium and low sodium, the adrenalin injections were followed by very toxic manifestations. In some cases it was necessary to institute treatment with cortical extract and intravenous saline infusions to save the animals. Early deaths occurred among such adrenalectomized animals if treatment was not resorted to soon enough. This observation is important in view of the undesirable, sometimes dangerous, reactions induced by adrenalin when administered to patients with Addison's disease.

Some of the results of experiments on 2 completely adrenalectomized animals in the series were selected for Table II, to illustrate the influence of physiological quantities of adrenalin in dogs deprived of both cortex and medulla of the adrenals. Both animals show the adrenalin effect to be the same as in normal dogs or in those in which only the epinephrine secretion from the medulla was interfered with. Dog "A" survived only a few hours after the adrenalin was administered, although he appeared to be in excellent condition at the beginning of the experiment. Dog "B" illustrates the lack of relationship between the levels of sodium and potassium when both are showing changes. In this animal life was prolonged by administration of interrenalin. One observation during that period is given and two observations that were made after hormone treatment was discontinued. The dog survived 10 days after cessation of treatment. In both tables, the dosage of adrenalin is given as μg per kg of body weight per minute. The amounts of Na and K are expressed in milliequivalents per liter of blood serum. Where two results are listed in the column under "During" adrenalin injection, the first represents a specimen obtained 2-3 minutes after beginning of the injection; the others were obtained about 45 minutes after beginning of the injection.

Inasmuch as reduction or suppression of epinephrine secretion from the adrenal glands causes not more than a moderate and transient elevation of blood potassium while complete adrenalectomy is generally followed by a persistent and more marked rise, the suggestion made previously, that the medulla rather than the cortex of the gland might be concerned with regulation of blood potassium, may be considered debatable. However, it is possible that an influence upon regulation of potassium in the blood can be exercised by epinephrine secretion from the medulla, loss of which can be compensated for by the function of other mechanisms. It is conceivable that such a compensatory mechanism, or mechanisms, could be so affected by the profound metabolic disturbance which results from loss of cortical function that the ability of the organism to maintain a normal level of blood potassium is lost. We believe that the foregoing evidence, in support of the possibility that regulation of blood potassium may be accomplished by functional activity of the adrenal medulla, is at least as credible as that which attributes such a function to the cortex.

Summary. 1. The physiological role of the adrenal glands in relation to blood potassium, hitherto attributed to function of the cortex or one of its steroid components, can be ascribed to a function of epinephrine secretion from the medulla.

2. The level of potassium in the blood serum is diminished by intravenous administration of adrenalin in such amounts and rate of injection as correspond with the physiological rate of epinephrine secretion from the adrenal glands.

3. This effect is obtained in normal dogs and in animals with reduced or suppressed epinephrine secretion. The elevation of blood potassium, which occurs in adrenalectomized dogs, can be corrected by constant intravenous injection of physiological dosage of adrenalin.

4. Higher dosage may effect a rise in serum potassium. A moderate increase in dosage usually induces a preliminary rise followed by a more lasting reduction in the level of potassium. This can also result from too rapid injection of smaller doses.

5. Significant changes in sodium were not constant. The supposed dependence upon the adrenal cortex for maintenance of a physiological equilibrium between sodium and potassium is not supported by the results of our experiments.

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Effect of Cholesterol on Antigenicity of Streptolysin O.* (17613)

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The hemolysins contained in culture filtrates of hemolytic streptococci have provided abundant research material since their discovery by Marmorek(1). Despite initial controversy, the existence of 2 distinct varieties of hemolysin is now well established, Todd(2), Barnard and Todd(3), Herbert and Todd(4), Herbert and Todd(5). The oxygen labile streptolysin O has received much attention in the literature, and among other interesting properties is the neutralization of its hemolytic effects by suspensions of cholesterol in high dilution, Hewitt and Todd(6). Cohen and Schwachman(7) noted that pneu-

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mococcus hemolysin, whose properties are similar to those of streptolysin O, was also neutralized by cholesterol. More recently Fisher(8) has noted that cholesterol and other sterols inhibit the hemagglutinins of *Hemophilus pertussis*. Saponin and digitonin, both of which are actively hemolytic, are inactivated by suspensions of cholesterol and plasma, Ponder(9). Hewitt and Todd(6) also reported that the intraperitoneal injection of sufficient cholesterol protected mice against subsequent lethal intravenous doses of streptolysin O. Although their observations were confined to a few mouse protection tests, this finding suggested that there could be *in vivo* neutralization of streptolysin O by cholesterol. They recorded no data on the effect of cholesterol on the behavior of streptolysin O as an antigen. The experiments reported here were conducted to determine the effect of cholesterol on the antigenicity of streptolysin O.

Materials and methods. Streptolysin O. Actively hemolytic culture filtrates were obtained from strain Richards grown overnight in Todd-Hewitt broth(10). Purification of the filtrate was taken to stage two of the method described by Herbert and Todd(4) and then lyophilized. The material was standardized by titrating against a standard serum following the method given by Todd(2) in Appendix 1. The material used in the majority of the experiments contained one unit of streptolysin O in 0.8 mg.

Cholesterol suspensions. Suspensions were prepared by dissolving U.S.P. cholesterol in a small quantity of 95% ethyl alcohol, adding an equal volume of 5% sodium oleate and adding dropwise to hot saline with constant shaking. By this means sufficiently smooth suspensions were obtained. Dilutions of these cholesterol suspensions in the region of 10^{-6} to 10^{-7} effectively neutralized one unit of streptolysin O. Hemolysis which might be due to sodium oleate was diluted out at this concentration.

Standard Antistreptolysin O. The antistreptolysin used consisted of refined serum globulins from hyper-immunized horses, prepared by E. W. Todd, and employed by many investigators as an arbitrary standard for antistreptolysin titrations. The sample used contained 20,000 units per ml and 0.35% trikresol as preservative. All dilutions were made in 0.85% sodium chloride solution.

Experimental. Titrations were carried out by mixing 1.0 ml volumes of a descending series of tenfold dilutions of cholesterol with 0.5 ml volumes of streptolysin O containing one unit in 0.5 ml. Active streptolysin O solution was prepared in saline containing 0.2% sodium thioglycollate as a reducing agent. After allowing 15 minutes in the water bath at 37° for neutralization to take place, 0.5 ml of a 3% washed rabbit red cell suspension was added and incubation continued for one hour. From these experiments the dilution of cholesterol suspension required to neutralize one unit of streptolysin O was determined. Mixtures of streptolysin O and cholesterol were prepared, in which 10 times the required amount of cholesterol was added. These over-neutralized mixtures were incubated in the water bath at 37° for 15 minutes and then inoculated intravenously into 2 rabbits. At the same time a mixture was prepared containing the same amount of streptolysin O but 10 times the required amount of standard antistreptolysin O. This too was incubated in the water bath at 37° before intravenous inoculation into a second pair of rabbits. Finally, streptolysin O alone was given to another pair of rabbits after having been incubated in the same manner. All rabbits were males weighing approximately 2 kg. The volume of all the inocula was 4.0 ml. The cholesterol in the streptolysin O mixture gave excellent dispersion, probably due to the protein present.

The animals were bled prior to immunization, and one week following the last dose. Two courses of immunization injections were given to each of the 3 groups of animals. The first course consisted of 50 units of streptolysin O, 50 units of streptolysin O plus 0.5 mg of cholesterol, 50 units of streptolysin O plus 500 units of antistreptolysin O to the

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TABLE I.
Antistreptolysin Titers in Immunized Rabbits.

	Streptolysin O alone		Streptolysin O + cholesterol		Streptolysin O + antistreptolysin O	
Rabbit	A_1	A_2	B_1	B_2	C_1	C_2
Before immunization	<1	<1	<1	<1	<1	<1
After first course	60	50	50	20	<1	<1
After second course	50	16	30	20	1	2

respective groups. After 4 days this dosage was doubled and after a further 4 days, 4 times the original dosage was given. After an 18 day rest period a second series of injections were given and bleedings taken as before. This consisted of 3 injections at 4 day intervals of the original doses. Antistreptolysin titres of all the sera were determined by the method outlined by Todd. All values are recorded with reference to the standard serum.

Results. Table I shows the antistreptolysin titres of the rabbits before and after each course of injections. It will be seen that the titres of antistreptolysin produced by the inoculation of streptolysin-cholesterol mixtures do not differ significantly from those produced by streptolysin alone. The inoculation of streptolysin-antistreptolysin mixtures produced no change in the antistreptolysin titre whatever after the first course of injections and very little after the second.

Discussion. The results indicate that the antigenicity of streptolysin O is not impaired when its hemolytic activity is inhibited by cholesterol. The explanation offered is that some easily dissociated streptolysin-cholesterol complex is formed which loses its ability to lyse red cells but still functions as an antigen. The chemical grouping by which cholesterol combines with streptolysin is a matter for further study although there is evidence which implicates certain regions on the cholesterol molecule(8). Hewitt and Todd point out that sulfhydryl groups added in the form of cysteine did not effect the neutralization of streptolysin O by cholesterol. We have found that oxidized, inactive streptolysin O is neutralized in the same way as the active reduced form. The addition of -SH containing reducing agents to mixtures of oxidized, inactive streptolysin O and cholesterol failed to restore any hemolytic activity except to tubes containing less than a neutralizing dose of cholesterol.

Summary. 1. The observation of Hewitt and Todd that cholesterol neutralizes the hemolytic activity of streptolysin O is confirmed.

2. The antigenicity of streptolysin O remains unchanged in cholesterol-neutralized mixtures.

3. Under similar conditions streptolysin O neutralized by its specific antibody elicits virtually no immune response to streptolysin O.

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Bioassay of Desoxycorticosterone-like Material in Urine.* (17614)

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Many explanations have been offered for the retention of sodium which is characteristic of the various edematous states. Injury,

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* The assistance of Mrs. Julia Harvey, Mr. William Lew, and Mr. Lee J. Poo is gratefully acknowledged.

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The crystalline hormones were a gift from Dr. Edward Henderson of the Schering Corporation.

ischemia(1), or congestion(2) of the kidney can reduce sodium excretion, while edema may also result from injury of capillaries or an alteration in hydrostatic-osmotic balance. The difficulty in explaining fully the presence and extent of edema by measurements of renal function, colloid osmotic pressure and venous pressure, has led to the suggestion that hormonal effects on the kidney might be important. Evidence of increased adrenal function in the edema of heart failure and of pre-eclampsia has been reported, but its relation to sodium retention is not clear(3,4,). In this communication, a method for estimation of sodium-retaining activity of urinary extracts is described, and some preliminary studies in edematous patients are reported.

Bio-assay method. Female adrenalectomized rats weighing 150 g are maintained for 3-4 days after operation on stock diet and given physiological saline to drink. On the day before the test, a sodium-free diet and distilled water are substituted, and each rat is given 5 cc of physiological saline intraperitoneally, which is repeated each evening during the test period. On the morning of the test, each rat is given 5 cc of distilled water intraperitoneally. Water intoxication is not seen, and the assay is more sensitive than when physiological saline is given. Urine is collected for 5 hours in individual metabolism cages. Urinary volume is recorded and total sodium excretion is measured with a flame photometer.

The first day serves as a control for subsequent test materials, which are injected 30 minutes before the beginning of the collection period. Rats with a sodium output below 30 μ eq on the control day are eliminated. If a rat is cold or lethargic at the end of a test, or if urinary output falls below 0.3 cc, the rat is discarded. The ratio of sodium excretion on the control day to that on the test day is used as a measure of activity of injected material. Repeated control tests on the second or third day in 5 groups of rats have given ratios of 0.7 to 1.2, averaging 1.0. Individual variations of response make it desirable to use groups of at least 5, and preferably 10 rats. Factors are averaged logarithmically.

This method follows the principles of Dorfman's(5) method, which we found inconvenient because of certain problems of supply, variable specific activity, and measurement of radioactive sodium, as well as because of variations from day to day due to the uncontrolled sodium intake.

Response to Crystalline Hormones. When a single rat group is given increasing doses of DOCA on succeeding days (Table I), a consistently increasing retention of sodium is elicited. However, the response to a standard dose of DOC or DOCA, as shown, varies between different rat groups. It is evident from the data that in order to obtain an accurate assay, a known dose of DOCA would have to be given to each group of rats.† Roughly, a factor greater than 2 may be expected with dosage of the order of 5 γ per rat. The factor of sodium retention increases with dosage, but a dosage of about 25 γ produces a maximum response. Estradiol, progesterone and testosterone have been assayed by this technic. In order to obtain a response comparable to the response to small doses of DOCA, the dose of these sex hormones must be 25 to 50 times greater. The order of effectiveness of these compounds (Table I) differs from that shown by Thorn and Engel(6) in male dogs.

Extraction and vehicles. Urine is acidified (pH 1.0) with HCl and extracted with chloroform. The extract is dried over sodium sulfate and evaporated to dryness *in vacuo* at 40°C. The residue is dissolved in ethyl alcohol, taken again to dryness, and redissolved in the proper volume for injection. The choice

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5. Dorfman, R. I., Potts, A. M., and Feil, M. L., *Endocrinol.*, 1947, v41, 464.

† A. G. Spencer (personal communication) has independently developed a similar method of assay of DOCA, using adrenalectomized mice, in which the control of each group with a known dose of DOCA has minimized the errors due to variation between groups.

6. Thorn, G. W., and Engel, L., *J. Exp. Med.*, 1938, v68, 299.

TABLE I.
Effect of Certain Steroids on Urinary Sodium Excretion of Adrenalectomized Rats.

Material injected	Dose, γ	Rats, No.	Sodium retention factor
Controls	—	5 groups of 5 rats	0.7-1.2 (mean 1.0)
Desoxycorticosterone	0.2	11	1.2
	0.5	4	1.0
	1	8	0.8
	1	5	0.5
	10	6	5.6
	25	5	4.5
Desoxycorticosterone acetate	2	9	1.7 a
	5	9	3.0 a
	10	9	3.1 b
	10	5	2.0 c
	25	9	4.3 b
	25	5	3.0 c
	50	9	4.2 a
	100	9	3.9 b
Estradiol	10	9	1.0 d
	50	9	1.3 d
	250	9	1.5 d
Progesterone	10	9	1.4 e
	50	9	1.6 e
	250	9	2.5 e
Testosterone	10	9	1.2 f
	50	9	1.5 f
	250	8	2.9 f

Factors followed by the same letter were obtained from the same group of rats.

of vehicle for injection is important. Peanut and sesame oils are poor solvents, and may retain enough chloroform to cause renal damage. Glycerol and propylene glycol are viscous and poor solvents, which in mixtures with alcohol cause sodium retention in doses used. Carbowax 1500 and 4000 cause renal injury and sodium retention. Carbitol did not lead to sodium retention, but at autopsy the kidneys were swollen and mottled. Ethanol and polyethylene glycol are effective solvents and cause no sodium retention. Ethanol has been selected for routine use because it is a less viscous, better solvent, and because a similar dose of DOCA produced a greater sodium retention under the experimental conditions when dissolved in ethanol than when dissolved in polyethylene glycol.

Urine extracts. Urine is collected as a 24-hour specimen to avoid diurnal variations(7), suggested by case 12 Table II. The dose is expressed as the number of minutes of urinary output represented by the extract administered to each rat. In 12 determinations on normal individuals and pools, the average factor is 1.25, with a range of 0.7 to 1.6 (Table II). In 10 determinations on non-edematous patients in the hospital, the average factor is 1.4, with a range of 0.7 to 2.1. An interesting feature is the failure of the factor to increase with increasing dosage. A maximum effect seems to be reached at about the 20-minute dose. In several instances, the decreasing response to increasing dosage suggests the presence of an interfering factor. Of the 5 cases with congestive heart failure, 4 cases gave results well above the normal range at the 20-minute dose, while the fifth case gave an unusually high result at a 40-minute dose. The extract from one of these patients after a mercury-induced diuresis was much less active. The patient with constrictive pericarditis showed no increase above the normal level. The extracts from 3 patients with

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7. Romanoff, L. P., Plager, J., and Pincus, G., *Endocrinol.*, 1949, v45, 10.

TABLE II.
Effect of Extracts of Human Urine on Urinary Sodium Excretion of Adrenalectomized Rats.

Group Case No. (Sex)	Description	Time dose (min.)	No. of rats	Sodium retention factor	Patient's urinary sodium (m.eq./24 hr)
Normals					
Pools		20	5	1.3*	
		20	5	0.7	
		20	5	1.4	
		120	4	1.3	
1 (M)		20	9	1.2	164
		40	10	1.2	
		120	6	0.9	
2 (M)		5	4	1.3	185
		20	9	1.6	
		40	10	1.5	
		120	8	1.1	
3 (F)	Immediately pre-catamenia	20	9	1.4	103
Cardiacs					
1 (M)	Arteriosclerotic heart disease in failure	20	5	1.7	11.5
		40	4	3.1	
2 (M)	Syphilitic aortic insufficiency in failure	20	4	2.1	2.1
3 (F)	Mitral stenosis, fibrillation, in failure	20	14	3.4	1.4
4 (M)	Tachycardia with decompensation	20	6	3.4	10
4 (M)	After mercurial diuresis	20	4	1.3	113
16 (M)	Arteriosclerotic heart disease, fibrillation, in failure	20	10	7.1	9 (Serum 127)
5 (F)	Constrictive pericarditis with edema	20	4	1.3	8
Nephrotic					
6 (M)	Edema-free	20	6	1.0	65
		60	5	1.3	
		120	6	0.6	
7 (M)	Moderate edema	20	15	1.6	1
		60	6	0.7	
		120	5	1.2	
8 (F)	Severe anasarca	20	9	6.5	0-1 (Serum 124)
Miscellaneous					
9 (M)	Acute glomerular nephritis, during diuresis	20	15	1.0	44
		60	4	1.2	
10 (F)	Probable lupus	20	10	0.7	189
		120	7	0.8	
11 (F)	Thyrotoxicosis	20	8	1.6	177
		110	9	1.7	
12 (M)	Hemophilia—daytime	20	8	1.6	76
12 (M)	" nighttime	20	9	1.3	71
13 (F)	Rheumatoid arthritis (pre-treatment)	20	10	1.9	57
13 (F)	After 3 days Δ-5-pregnenolone	20	8	2.7	70
14 (F)	One kidney. No edema. Midcycle	20	9	2.1	176
15 (M)	Addison's disease, receiving DOCA	20	8	1.7	103
		120	9	1.0	

* This extract administered in oil.

nephrosis varied from normal to nearly the highest activity observed in an order corresponding to the severity of their edema.

Discussion. It is obvious that the results with urine extracts cannot be interpreted at this stage. Fractionation of the extracts must be attempted in an effort to identify or characterize the active principle which appears to be present. By such a procedure it may also be possible to explain or eliminate the presently paradoxical time-dose response observed. The preliminary observations reported indicate the presence of sodium-retaining factors in the urine of some cardiac and nephrotic patients in concentration greater than that observed in normal controls or in patients with other forms of disease.

Summary. An assay for desoxycorticosterone-like activity in biological fluids is presented, having a sensitivity in the order of 10 γ of DOCA. The critical nature of solvent vehicle choice and of time-dosage of urinary extracts is presented and discussed. Urinary extracts from some edematous patients with heart failure and nephrosis show a sodium-retaining activity greater than that observed in normals and in non-edematous controls.

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Autotransplantation of Adrenal Gland in the Spleen and Mesentery of Rats. (17615)

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Ovarian transplants in the spleen survive and serve as an excellent tool in elucidating the role of the liver in gonadal hormone metabolism(1). Transplants of the adrenal gland in the spleen have hitherto been unsuccessful(2,3).* This paper reports the successful autotransplantation of the adrenal gland in the spleen and mesentery of the rat, with evidence that such a transplant permits normal maturation of the animal.

Experiments. In one experiment, each of 3 male and 3 female immature rats of the Long-Evans strain, averaging 62 g in weight, was subjected to 2 operations. In the first procedure, either a half or the entire left adrenal gland was transplanted into the spleen, except in 2 animals, where the ileocolic mesentery was employed. The second procedure consisted of right adrenalectomy. In the 3 females, this was done 8 days after the first operation, and the animals drank normal saline instead of water for the subsequent 8 days. Right adrenalectomy was performed in the 3 males 16 days after the initial transplantation, and tap water was the only fluid provided. Food was supplied *ad libitum* throughout the experiment. After 108 days, the 6 animals were sacrificed. They now averaged 240 g in weight and appeared normally developed when autopsied. In 5 of these 6 animals, the transplants varied from 3 to 5 mm in diameter, and were sharply outlined. On microscopic examination, they were composed of cells of the adrenal cortex. One of the 4 intrasplenic transplants could not be traced; of the other 3, a typical example is shown in Fig. 1A, B. Both of the ileocolic mesenteric transplants were successful; one of these shown in Fig. 2A, B.

In another experiment, 6 Long-Evans, adult, female rats were bilaterally adrenalectomized, and at the same time one of the adrenals was transplanted into the spleen. Two of the animals were sacrificed after 50 days and the remainder after 75 days. There was an average weight gain of 30 g and all of the transplants were successful. The transplants after 50 days were 4 mm in diameter and from 2-4 mm after 75 days. In every case they were composed of cells of the adrenal

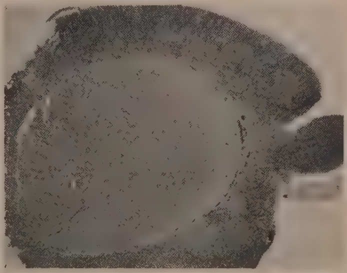

FIG. 1 A.
(S-3044 10× approximately) A 108-day-old transplant is well outlined in the splenic pulp.

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1. Biskind, M. S., and Shelesnyak, M. C., *Endocrinology*, 1942, v30, 819.

2. Oldberg, E., *Am. J. Physiol.*, 1929, v91, 275.

3. Grieco, F., *Arch. Ital. di Anat.*, 1932, v3, 589.

* Addendum: An apology is offered to E. O. Butcher for oversight of his report in *Endocrinology*, 1948, v43, 30.

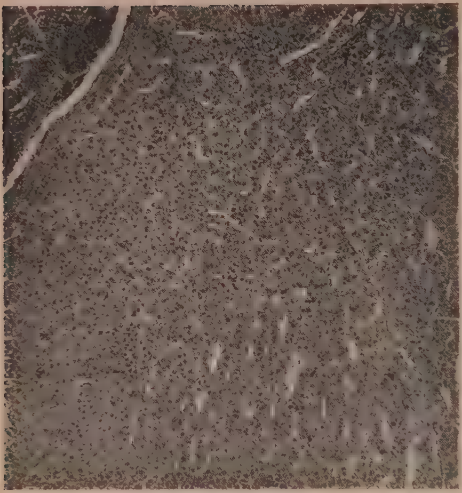

FIG. 1 B.
(S-3044 50× approximately) A higher magnification of 1 A demonstrates that the cells of the cortex are not demarcated into zones. No medullary tissue was found.

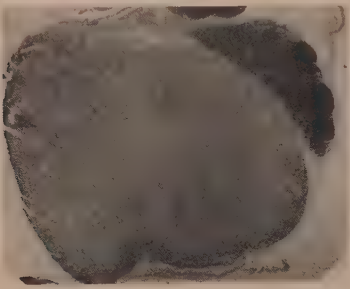

FIG. 2 A.
(S-3037 10× approximately) This adrenal transplant in the ileocolic mesentery is 108 days old and is derived from one-half of the left adrenal gland.

cortex, microscopically. In all of these animals, extensive abdominal adhesions were present, particularly around the spleen.

In 8 other adult rats, the left adrenal was transplanted to the spleen, and the right adrenal left *in situ*. Autopsy 92 days later revealed complete atrophy of the adrenal transplants in these animals.

Discussion. Since autopsy revealed no adhesions in 4 out of 5 successful transplantations of the adrenal gland in the immature rat, secretions from the splenic transplants could only be liberated into the portal circulation, and from the mesenteric transplants into the portal, or abdominal lymphatic circulations. If these transplants are composed of functioning tissue of the adrenal cortex, and if accessory adrenal cortical tissue is not responsible for the growth and maintenance of the animals, then it would indicate that the liver plays no essential destructive role in the metabolism of the adrenal cortical steroids. The control experiments in 8 animals in which the transplants atrophied when the right adrenal gland was left *in situ* indirectly suggests that the presence of functioning adrenal cortical tissue prevents successful transplantation. Since no medullary tissue was found in any of the successful transplants, it is suggested that such transplants are composed of regenerated adrenal cortical cells. In addition to the above work under investigation, reports will soon be forthcoming on embryonic homotransplantation and transplantation in castrates.

Summary 1. Autotransplantation of the adrenal gland in the spleen and mesentery of the rat was successful.

2. A technic is described which may aid in the elucidation of the role of liver in adrenal cortical metabolism.

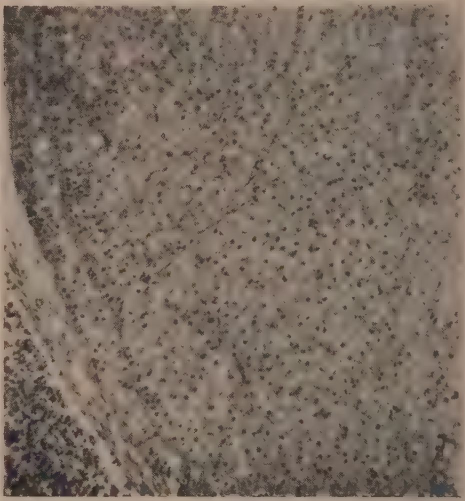

FIG. 2 B.
(S-3037 70× approximately) In a higher magnification of 2A the cells of the adrenal cortex are arranged in cords but without distinct zonal pattern. No medullary tissue was present.

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Plasma Progesterone Levels and the Menstrual Cycle of the Monkey.*†
(17616)

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Present concepts of the humoral environment of the endometrium through the menstrual cycle are almost entirely deductions from observations of the effects of artificially supplied steroid hormones, from studies of the structure of the endometrium and ovaries during the cycle, and from measurements of urinary steroids during the various phases of the cycle. With respect to progesterone, apparently the only reported attempts to ascertain blood levels have been those of Clauberg(1), Bloch(2), and de Allende(3). Clauberg was unable to detect progestational activity in extracts of human menstrual cycle blood and of human pregnancy serum and blood. Bloch found less than 1 Rabbit Unit of progesterone in 8 to 12 liters of human pregnancy blood. De Allende reported that blood samples taken at 3-day intervals throughout several menstrual cycles of monkeys assayed at from 0.06-0.12 μg to 0.25-2.5 μg/cc (McPhail scale). A method for the bioassay of progesterone has been developed(4) primarily with physiological measurements of this nature in mind. The method involves injection of serial dilutions of the material to be tested into the lumina of uterine segments of ovariectomized mice. The method regularly detects 0.0002 μg of progesterone, is apparently specific for progesterone(5), and can be applied to measurements of blood levels(6).

The present study was undertaken solely as a preliminary survey and with reference to several questions: is blood progesterone present in the pre-ovulatory phase of the cycle; what is the magnitude of blood level of progesterone in the luteal phase of the cycle; is the level uniform throughout the luteal phase; is the onset of menstruation preceded by a significant decline in the level of progesterone in the blood?

Each of 2 adult rhesus monkeys was studied through a cycle extending from the middle of May until mid-June. The 2 animals had exhibited cycles averaging 27.8 days (range, 26-29 days; monkey A42) and 28.6 days (range, 25-32 days; monkey A49), respectively, as revealed by daily vaginal smears through the several months preceding the assays of the blood. The dates when blood was obtained are shown in the chart. In each monkey the study began toward the end of one cycle and continued to approximately the same day in the succeeding cycle. The lengths of the cycles during the study are indicated on the chart. Points indicating assay values have been connected with straight lines solely to aid visualization; it should not be inferred that the lines indicate progesterone levels obtaining on the days on which blood samples were not taken. In each instance a one ml sample of blood was drawn from a saphenous vein with sodium citrate serving as the anticoagulant. The sample was promptly centrifuged, and the plasma progesterone was separated into the free and bound fractions as described earlier(6). (It has already been shown that progesterone is present in plasma

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* This study was aided by grants from the Committee for Research in Problems of Sex, National Research Council, and from the James Hudson Brown Memorial Fund of the Yale University School of Medicine.

† Dr. F. E. Bryans at the Harvard Biological Laboratories has independently completed a similar and more extensive study which will appear soon.

‡ Present address: Department of Anatomy, University of North Carolina School of Medicine, Chapel Hill, N.C.

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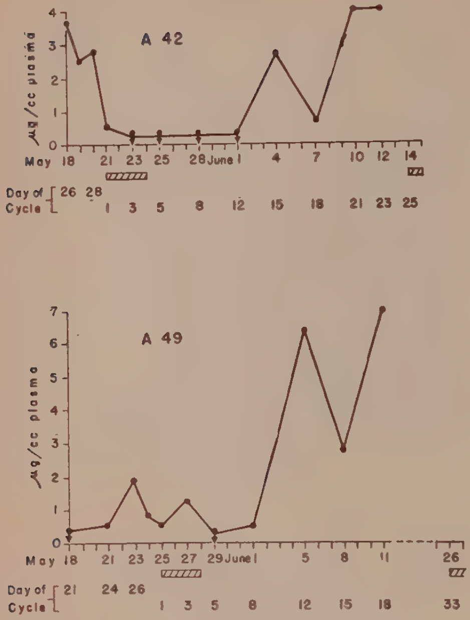

FIG. 1.

Free plasma progesterone levels in 2 monkeys through a menstrual cycle. In each instance the day of the cycle and the date are shown. The arrows signify that the values thus indicated are not known, but are less than those indicated by the solid circles.

and not in the blood cells)(6). The two fractions were incorporated in sesame oil, and dilutions were prepared so that the assay values obtained are believed to be accurate within a fraction of a μg per ml of original plasma.

The values for free plasma progesterone are shown in the chart. In general, the 2 animals showed parallel fluctuations in levels of progesterone. During menstruation and in the early days following menstruation the level was low. In each monkey, a relatively high value was present near the middle of the cycle, a decidedly lower level followed, and a high level appeared again in the latter half of the cycle. A low level was present at or preceding the onset of menstruation.

The low level during the first half of the cycle was expected on the basis of the conventional account of the menstrual cycle. The source of the small amounts of progesterone during this portion of the cycle in monkey A49 is unknown.

Presumably the high level near the middle of the cycle was related to ovulation, although it is not known when ovulation occurred in either animal, or indeed whether ovulation occurred. Moreover, it is not known whether the high level was brief or up to 4 days in duration. The two questions may prove to be highly significant in the analysis of the circumstances at and related to ovulation.

The values in the latter half of the cycle are interesting in relation to the hypothesis that menstruation is provoked by diminution in progesterone. In monkey A42 relatively high levels were found on the 3 days preceding the first menstruation. On the first day of menstruation the level had dropped to a low value. The values in this period of 4 days may indicate a steady decline preceding menstruation. On the other hand, the relatively small day to day differences on the 26th, 27th and 28th days of this cycle may be unrelated to each other or to menstruation. In the succeeding cycle high levels were found 4 and 2 days before a single day of vaginal bleeding. This bleeding, however, cannot be considered to be a typical menstruation inasmuch as a 3-day period of bleeding began 9 days later. Unfortunately, the earlier bleeding had been accepted as menstruation, and the blood levels of progesterone during the next 9 days were not followed. The progesterone levels on May 18 to 20 in this animal seem somewhat at variance with the general observation that experimental menstrual bleeding in ovariectomized monkeys begins approximately 3 days after the last of a series of injections of progesterone(7).

In the second animal, monkey A49, the level was inconstant but relatively low for at least 7 days before menstruation. This circumstance also seems not to be consistent with observations on experimental menstruation, but in the opposite direction. Here, however, it may be that the menstruation followed an

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7. Allen, E., Hisaw, F. L., and Gardner, W. U., 1939, Chap. 8 in Allen, E., Danforth, C. H., and Doisy, E. A., *Sex and Internal Secretions*, Baltimore, Williams and Wilkins.

anovulatory cycle. Hartman(8) has reported that the monkey ovulates infrequently during the summer months, and the menstruation occurred toward the end of May. If the cycle was in fact anovulatory, the source of the progesterone is unknown. The high level of progesterone found in the second cycle in this animal indicates that a corpus luteum must have been present. This cycle, however, was 32 days in length, and menstruation began on June 26, 14 days after the last assay. Consequently, the level of progesterone in relation to the onset of the second menstruation is unknown.

Single values in 3 other intact monkeys may be mentioned. In one animal a level between 7.7 and 8.9 μg per ml plasma was found 10 days before the onset of menstruation; in a second animal the level was 2.8 μg per ml 3 days before menstruation; in a third animal the level was 0.9 μg per ml 2 days before menstruation. It is clearly impossible to say whether these isolated values conform to any pattern shown by the 2 animals studied throughout a cycle. The values for bound plasma progesterone ranged from less than 0.2 to 0.4 μg per ml. The small fluctuations encountered seemed to be quite unrelated to the phase of the cycle

The uniformly low levels of bound progesterone are consistent with our other experience with this fraction. Its level has been low under all physiologic circumstances in several species(6). It has been high only in animals receiving injections or carrying pellets of progesterone; in the mouse the bound fraction was biologically inert, and binding was a means of hepatic inactivation of progesterone(9).

Summary. Bioassay of the free and bound plasma progesterone at various intervals through a menstrual cycle in each of 2 monkeys indicate the level of free progesterone to be low during and following menstruation, high near the middle of the cycle, lower following this peak, and high in the latter half of the cycle. A low level was present at or preceding menstruation. The levels of the bound fraction were low at all times.

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Survival of *Schistosoma mansoni in vitro*.* (17617)

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Information about the survival of schistosomes *in vitro* has been limited to the observation that *Schistosoma japonicum* can be maintained alive in serum for periods of 8 to 83 days(1,2). Similarly, we have found that *Schistosoma mansoni* survives for 14 to 18 days in horse serum. Knowledge about the nutritional requirements of these trematodes could be acquired by investigating the constituents of serum, essential for their survival. Accordingly, studies designed to define these factors were initiated using *Schistosoma mansoni*.

Methods. All experiments were conducted under aseptic conditions. Pairs of schistosomes

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* This investigation was supported by a research grant from the Division of Research Grants and Fellowships of the National Institutes of Health, U. S. Public Health Service.

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were removed, with dissecting needles, from the mesenteric and portal veins of mice infected 6 to 8 weeks previously by intraperitoneal injection of 160 cercariae per mouse. The helminths were washed in 5 to 10 ml of a medium which had the same composition as that used in the subsequent experiment. The parasites were transferred to Stender dishes containing, per pair of worms, 3 to 5 ml of the medium to be tested; incubation was carried out at 37°C. The medium was renewed every 3 days by replacement rather than by transfer of the schistosomes. Motility of the worms was used as the criterion of survival. Usually, this was easily detected. In questionable cases, the worms were considered dead if motility was absent in two separate observation periods (5 minutes), under the low power dissecting microscope. The chemically defined medium (SM), had the following composition per 100 ml: NaCl, 753 mg %; $CaCl_2$, 4.4 mg%; $NaNO_3$, 0.10 mg %; Na_2SO_4, 0.66 mg %; KCl, 1.73 mg %; $MgCl_2$, 50 mg %; $MnCl_2$, 5.4 mg %; $FeCl_2$, 0.04 mg %; tris(hydroxymethyl)aminomethane buffer (pH 8.0), 0.0025M; $NaHCO_3$, 0.0052M; Na orthophosphate buffer (pH 8.0), 0.0021M; DL-lysine, 7.8 mg %; DL-methionine, 6.5 mg %; DL-threonine, 6.5 mg %; DL-valine, 6.5 mg %; L-arginine HCl, 3.95 mg %; L-histidine HCl, 1.3 mg %; DL-isoleucine, 5.2 mg %; DL-phenylalanine, 2.5 mg %; L-leucine, 7.8 mg %; DL-tryptophane, 2.0 mg %; DL-alanine, 10 mg %; glutathione, 10 mg %; cysteine HCl, 0.10 mg %; p-aminobenzoic acid, 0.125 mg %; carotene, 0.01 mg %; ascorbic acid, 0.01 mg %; thiamine, 0.01 mg %; riboflavin, 0.02 mg %; niacin, 0.05 mg %; pyridoxine HCl, 0.10 mg %; inositol, 0.05 mg %; Ca pantothenate, 0.10 mg %; biotin, 0.0005 mg %; choline chloride, 0.05 mg %; folic acid, 0.0001 mg %; uracil 2 mg %; adenine sulfate, 2 mg %; xanthine, 2 mg %; hypoxanthine, 2 mg %; guanine, 0.4 mg %; glucose, 50 mg %. In this medium the schistosomes survived for only 12 to 18 hours.

Results. The schistosomes survived for almost the same length of time in serum ultrafiltrate (UF) (10 to 12 days) as in whole serum (14 to 18 days). This indicates that protein and other non-dialyzable components of serum are of little significance to the survival of the worms. The pH of individual batches of serum ultrafiltrate varied considerably (7.8 to 9.5). Accordingly, attempts were made to stabilize the pH of the UF. The CO_2 contained in the UF was driven off by aeration for 10 minutes at pH 4.0. The pH was then adjusted to 8.0 with NaOH. This solution was sterilized by filtration through a sintered glass filter (Corning Glass No. 330990). One ml of 0.5M $NaHCO_3$ and 1.25 ml of 0.2M tris(hydroxymethyl)aminomethane buffer (pH 8.0)(3) per 100 ml were added to this serum ultrafiltrate immediately before each experiment. No difference in the duration of survival of the worms was observed regardless of whether they were incubated in untreated UF or in UF treated in this manner. A twofold increase in the bicarbonate concentration reduced the survival time. Orthophosphate (0.005M) and pyrophosphate (0.005M) buffers proved highly toxic, allowing survival for not more than one day. The brief survival of the worms in SM was due to a nutritional inadequacy rather than to a toxic effect, since addition of all the constituents of this medium (with the exception of NaCl) to UF, in concentrations similar to those used in SM, did not shorten the period of survival of the organisms. However, sodium nitrate and sodium sulfate in a concentration of 5 mg % proved toxic to the worms. Addition to SM of the following materials failed to increase the period of survival of the worms: ashed ultrafiltrate, $MnCl_2$, glutamine, acid or enzymatic casein hydrolysates, ornithine, creatine, creatinine, urea, Tween 80, beta-hy-

TABLE I.
Survival of *S. mansoni* in SM Supplemented with Protogen.

Protogen units per ml	Survival in days
2000	9
90	4
60	1
30	1
15	<1

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TABLE II.
Survival of *Schistosoma mansoni* and *Litomosoides carinii* Under Aerobic and Anaerobic Conditions.

Helminth	Medium	Atmosphere	NaCN	Survival (days)
S. mansoni	UF	Air	—	12
” ”	UF	100% N_2	—	5
” ”	UF	Air	1×10^{-3} M	6
” ”	Ox Serum	”	—	16
” ”	” ”	100% N_2	—	5
Litomosoides carinii	” ”	Air	—	7
” ”	” ”	100% N_2	—	< 1

droxybutyric acid, citrate, ribose, xylose, ribonucleic acid, desoxyribonucleic acid, a concentrate of strepogenin,† vitamin B_{12}, a concentrate of animal protein factor,‡ thymine, pyridoxal. In a medium containing 50% UF and the same concentrations of NaCl and glucose as are present in SM, survival was as long as in undiluted UF. However, a further reduction of UF to a concentration of 25% permitted survival of the worms for only 3 days instead of 11 days. Addition of an aqueous extract of muscle (Difco No. B126) to SM resulted in a marked increase in survival time. If SM contained 25 mg/ml of this extract, survival of the worms almost equalled that observed with undiluted ultrafiltrate. The factor or factors in this muscle extract responsible for survival were found to be soluble in 35% and insoluble in 90% ethyl alcohol. A significant increase in the periods of survival occurred also when a fraction of purified protogen(4,5) (NP-41-160-1-H-0)‡ was added to SM (Table I).

S. mansoni can survive in the host under conditions that produce a marked reduction in the respiratory metabolism of the fluke(6,7). Furthermore, this parasite utilizes little or no energy liberated from oxidative metabolism(8). Additional evidence that respiratory metabolism is of minor significance to survival is supplied by the fact that the schistosomes can survive (Table II) in 100% nitrogen for at least 5 days. Similarly, survival for 5 days occurred in 1×10^{-3}M cyanide (under conditions where loss of HCN was precluded). A complete inhibition of respiration was observed in worms which had been incubated for 4 days in cyanide 1×10^{-3}M), while their rate of aerobic and anaerobic glycolysis was the same as in controls not exposed to cyanide. Since in similar media, survival is more than twice as long aerobically as anaerobically, a critical requirement for oxidative metabolism must develop during or at the end of 5 days. By contrast, the filarial worm, *Litomosoides carinii,* did not survive under anaerobic conditions for more than 12 hours. This helminth has a high requirement for oxidative metabolism and partial inhibition of its oxygen uptake in the host results in its death(9,10).

Summary. 1. A method is described for studying the survival of *S. mansoni in vitro*. 2. These parasites survived in serum for 14 to 18 days; in serum ultrafiltrate for 10 to 12 days. 3. In a chemically defined medium, the survival of the parasite did not exceed 18 hours. 4. Addition of an aqueous extract of beef muscle or of a purified fraction of protogen to the synthetic medium significant-

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† Kindly supplied by Dr. D. W. Woolley.

‡ Kindly supplied by Drs. T. H. Jukes and E. L. R. Stokstad, Lederle Laboratories Division, American Cyanamid Company, Pearl River, N. Y.

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ly increased the period of survival. 5. Under completely anaerobic conditions the schistosomes survived for 5 days.

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Changes in Carcass Urea Following Evisceration in the Rat. (17618)

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The generally accepted conclusion that liver is the only mammalian tissue that can synthesize urea is based in major part upon the classical studies of Bollman, Mann and Magath(1) who noted that the level of blood urea remained steady following hepatectomy-nephrectomy in the dog, of Bollman and Mann(2) who found no evidence for the conversion of ammonia to urea in the liverless dog, and of Krebs and Henseleit(3) who found that the liver slice was the only tissue which could convert ammonia into urea. Maddock and Svedberg(4) found no evidence for urea synthesis in the liverless monkey. Observations upon the liverless animal have been limited to a few species and to survival periods of a few hours. We have studied changes in the blood and tissues of eviscerate rats that survive for periods of 48 hours and longer. Ingle, Prestrud and Nezamis(5) noted that the blood urea of the rat infused with solutions of glucose and insulin did not fall during the first 24 hours following evisceration although the kidney continued to excrete urea and the animal was in positive water balance, both of which might be expected to lower the urea of the body-water in the absence of urea synthesis. Since the circulation and water metabolism of the eviscerate rat are greatly changed from normal, it would be unsafe to assume that the urea content of its blood is representative of its distribution in the body water or that these observations on the rat provide valid evidence for the extra-hepatic synthesis of urea.

We have reinvestigated the problem by determining the urea content of the carcass and urines of rats at the time of evisceration and of similar rats 24 and 48 hours following evisceration. There was a decrease in the total urea of the carcass and urine following evisceration.

Methods. Male rats of the Sprague-Dawley strain were fed Archer Dog Pellets. At attainment of a weight of 185 to 205 g, the inferior vena cava was ligated between the liver and kidneys in order to cause the development of a collateral circulation. When the animals reached a weight of 250 ± 2 g they were anesthetized with cyclopal and eviscerated by the method of Ingle(6). Asepsis was preserved in both stages of the operation. A solution containing glucose, insulin, and 0.9% sodium chloride was infused into the saphenous vein of the right hind leg by means of a constant injection machine which delivered fluid from each of 6 syringes at the rate of 20 cc in 24 hours. The glucose load was 44 mg per 100 grams of rat per hour and 4 units of regular insulin (Lilly) per rat per 24 hours. Temperature was constant at 26.5°C.

The total urea of the carcass was determined as follows: The tail of the eviscerate rat was cut into small pieces and placed in

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TABLE I.
Total Carcass Urea of Eviscerate Rats. Means and Standard Errors of the Means and Differences Between Means.

Hr following evisceration	No. rats	Total urea, mg	Difference from controls
0	26	203.2 ± 6.3	
24	24	185.6 ± 4.6	17.6 ± 7.8
48	16	180.5 ± 7.7	22.7 ± 10.0
24- and 48-hr groups combined	40	183.3 ± 4.1	19.9 ± 7.5

the body cavity. The carcass was chilled in a deep freeze for one hour. Freezing of the carcass was avoided since it hindered grinding. The carcass was then passed twice through a power grinder which had been cooled by grinding dry ice in it. It was necessary to remove skin from the blade after the first grinding and to cut it into smaller pieces. Finely powdered dry ice was sprinkled over the tissue whenever a delay occurred. The ground tissue was transferred to a Waring blendor containing 150 cc of chilled saline plus the urine excreted by the rat following evisceration. After an initial stirring, 150 cc more saline was added, the blendor covered, and the mixture homogenized for 2 minutes. Two 50 cc aliquots were each added to 400 cc N/12 H_2SO_4 in 2-liter Erlenmyer flasks and allowed to stand one hour with occasional shaking. To each flask was added 50 cc of 10% sodium tungstate, the flask stoppered, the contents shaken and allowed to stand 30 minutes before filtering. The remaining homogenate was measured and the total volume recorded. The Folin-Wu filtrate was analyzed for urea by gasometric measurement of the carbon dioxide formed by the action of urease according to the procedure of Van Slyke(7).

Experiments and results. The total carcass urea was determined on 26 rats immediately following evisceration, on 24 rats that were infused for 24 hours following evisceration, and on 16 rats that were infused for 48 hours following evisceration. All of the urine excreted by each rat following evisceration was added to the carcass at the time of homogenization. The data are summarized in Table I. When the 24 and 48 hour groups are combined and compared with the control animals, the ratio between the difference in means and the standard error of the difference is 2.65. From the statistical standpoint, the chances that there was a true decrease in total urea following evisceration are greater than 99 in 100. The acceptance of statistical analyses of data requires the assumption that all of the uncontrolled variables which affected the results were randomized. We are not aware of any incidental errors or of any extra-renal means whereby urea could have been lost from the carcass, but such may have existed.

The acceptance of this evidence that urea disappears from the carcass of the eviscerate rat to a greater extent than can be accounted for by urinary excretion seems to require the conclusion that urea can be metabolized in the absence of the liver. Bollman and Mann(2) observed that the administration of urea to the hepatectomized dog caused a rise in urinary ammonia, thereby adding support to several lines of indirect evidence that the kidney can convert urea to ammonia. The studies relating to the origin of ammonia have been reviewed by Peters and Van Slyke(8) who concluded that urea is not a precursor of ammonia. Leifer, Roth and Hempelmann(9) have reported that the carbon of urea labeled with C_{14} is excreted in the expired air of the normal rat. This observation does not prove the catabolism of urea since there may have been an exchange of carbon between urea and CO_2 without a decrease in total urea. Rose, Smith, Wormack and Shane(10) have shown that urea can be

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utilized for the synthesis of non-essential amino acids by the intact young rat. The possibility that urea can be utilized in anabolic processes in the eviscerate rat should be considered.

The possibility that urea can be metabolized in the eviscerate rat weakens one point of evidence that urea synthesis occurs only in the liver. The earlier studies(1) on nephrectomized-hepatectomized animals have shown that the blood and tissue urea did not change during the times that these observations were made. The assumption that urea could not be metabolized therefore required the conclusion that no extra-hepatic synthesis of urea could have occurred since the concentration in the body fluids remained unchanged. However, if urea can be metabolized, it is hypothetically possible for extra-hepatic urea synthesis to coexist with an equal or higher rate of urea metabolism without increasing the concentration of urea in the body. As an analogy, it was first assumed that gluconeogenesis occurs only in the liver because the blood glucose falls following hepatectomy. It has now been clearly established that glucose can be formed in the kidney, but that this extra-hepatic gluconeogenesis is masked by a higher rate of glucose utilization(11). Evidence has been reported by Graham, Houchin and Turner(12) that the active mammary gland of the goat can synthesize urea.

Summary. Male rats of the Sprague-Dawley strain were eviscerated at a weight of 250 g and were given intravenous infusions of solutions of glucose, insulin and 0.9% sodium chloride for periods up to 48 hours. Comparisons were made of the total carcass plus urinary urea in 26 control rats taken immediately following evisceration, 24 rats which were killed 24 hours following evisceration and 16 rats which were killed 48 hours following evisceration. The average total carcass urea of the 24-hour rats was lower than that of the controls, and the average value for the 48-hour group was still lower. From the statistical standpoint, it is highly probable that there was some extra-renal loss of urea following evisceration. These results are indirect evidence for the occurrence of urea catabolism. The results are in agreement with observations on other species in showing that there is no rise in the urea concentration of the body in the absence of the liver.

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Increased Tolerance of Mice to Lethal X-Radiation as a Result of Previous Sublethal Exposures.* (17619)

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The pathogenesis of acute radiation illness is poorly understood. The discovery of factors that may influence the survival after exposure to doses of radiation in the lethal range will help considerably in the understanding of the mechanisms that produce this illness. The release of heparin and histamine in irradiated animals has been reported(1,2,3). This suggests an anaphylactoid response. In fact a similarity between hypersensitive states and

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[*] The opinions or assertions contained herein are the private ones of the authors and are not to be construed as official or reflecting the views of the Navy Department or the naval service at large.

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TABLE I.
Tabulation of Comparative Mortality and Weight Data on 3 Groups of Mice (100 mice each) Showing That Mice Previously Exposed to Fractional Sublethal Doses of X-ray Radiation Have an Increased Tolerance to a Subsequent Lethal Dose of X-ray Radiation.

Group	Comparative groups of mice	28-day mortality %	Days after exposure for max. wt loss	Wt loss or gain in % of original wt	Avg survival time, days
1. Irrad. control	703 r	41	14	—14.4	14.3
2. ” ”	144 r × 3	0	14*	— 1.6	
3. ” ”	144 r × 3, 703 r	26	14.5	—13	15.1

$0.05\ P_{diff}\ 0.02.$
* Fourteen days after the last sublethal exposure.

the toxicity of ionizing radiation has been suggested(4,5).

The natural course of acute radiation illness induced by total body exposure to ionizing radiation suggests that sensitization phenomena may participate in provoking the exacerbation in the severity of the illness that terminates the latent period(6,7,8). Of great interest is the report of Raper in which the LD_{50} of total surface beta radiation was increased by a previous sublethal cutaneous exposure to beta rays(9). With these facts in mind it was decided to try to alter the lethality of total body x-ray exposure on mice by previous fractional exposure to sublethal total body x-ray radiation.

Materials and Methods. White, Swiss, female mice raised at this Institute by brother-sister matings were used exclusively. Mice were obtained from stock at 30 days of age, observed for 2 weeks and then irradiated by the angular beam of a 1,000 KV, 3 ma, GE Industrial X-ray machine(10,11). The experiment utilized 3 groups of mice. Group 1 received no preliminary radiation. Groups 2 and 3 were irradiated according to the following schedule: A sublethal dose of 144 r was given at weekly intervals for 3 weeks. Thirty days after the third weekly dose Groups 1 and 3 were given a lethal range dose of 703 r simultaneously. All x-ray radiation was delivered at the rate of 31.2 r/min. Animals were checked 2 times per day and deaths recorded. Other studies, except weighing, which was done as a group without handling, were omitted so that extraneous factors such as handling and bleeding would not influence mortality. All mice were given Purina Mouse Chow and water *ad lib.*

Results. In Group 1, the controls, the first deaths occurred on the ninth day after the exposure to 703 r. Most of the deaths occurred between the ninth and the twenty-first days. The twenty-eighth day mortality was 41%. In Group 2, the mice that received only the 3 initial sublethal exposures of 144 r each there were no deaths. These animals appeared healthy at all times. However, there was a slight weight loss following each initial sublethal exposure in Groups 2 and 3. This was practically regained by the time each subsequent exposure was given and all weight loss was regained at the end of the 30 day rest period. Group 3 which received 144 r at weekly intervals for three weeks and then 703 r had an ultimate 28 day mortality of 26%. The probability that the difference in mortality between Groups 1 and 3 is due to chance was calculated by Chi square and found to be between 0.02 and 0.05 (Table I).

Comments. It is difficult to understand the mechanism by which previous sublethal ex-

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posures presumably increase resistance to radiation. It may be that the non-specific resistance to stress as defined by Selye(12) is increased. Perhaps specific resistance to the products of cellular destruction is increased comparable to the increased resistance of rabbits to bacterial toxins produced by previous x-irradiation as described by Bisgard(13). The results reported here for total body x-irradiation are similar to those reported by Raper(9) for total body-surface Beta radiation of mice.

Summary and Conclusion. 1. It has been demonstrated with a fair degree of certainty ($0.05 > P_{diff} > 0.02$) that three weekly exposures of 144 r followed by a 30 day rest period increases the resistance of mice to a subsequent lethal dose of x-radiation.

2. It is concluded that no adequate explanation for this phenomenon of increased tolerance to x-ray radiation is available at the present time.

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Phytotoxic Properties of Spinal Fluids from Paresis, Tabes and Neuro-Syphilis. (17620)

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The author has been studying the pharmacological properties of bloods from various psychoses for many years(1,2). The findings established clearly that sera from patients with organic and functional psychoses when tested by the author's standardized phyto-pharmacological methods, exhibited marked toxic effects on the root-growth of *Lupinus albus* seedlings grown in plant physiological solutions. The sera from purely psychoneurotic patients were not phytotoxic. More recently, the writer has begun a phyto-pharmacological examination of normal and pathological spinal fluids by exactly the same methods as those employed for examination of blood sera. In the present paper are reported the findings with the spinal fluids from a series of cases with general paresis and a series of non-psychotic patients suffering from Tabes Dorsalis and other cases of neuro-syphilis.

The methods employed have been described(3,4,5). The effect of spinal fluids dissolved in Shive's plant physiological solution(6), was studied quantitatively on the root-growth of *Lupinus albus* seedlings for 24 hours under standardized ecological conditions. By the index of growth is meant the ratio of the root-growth in a solution of the unknown drug to the growth of control seedlings in normal physiological solutions.

$$\text{Index of growth} = \frac{X}{N} \times 100$$

It was found that 1% solutions of normal spinal fluids exert practically no phytotoxic effect on the root-growth. A 2% solution of spinal fluid gives an average index of growth of 92%, the readings ranging from 80% to 100%. Table I shows the results obtained with a series of 16 cases of paresis, 10 cases of tabes and 14 cases of other neuro-syphilitic patients. It will be seen that all of the paretic spinal fluids yielded readings far be-

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TABLE I.
200 Normal Human Spinal Fluids 2% in Shive's Sol. Give Average Phytotoxic Index of 92%.

Patient with general paresis	Index of growth %	Patient with tabes dorsalis	Index of growth %	Patient	Disease	Index of growth %
J.L.	55	S.1.	84	S.7	Neurosyphilis unclassified	78
A.W.	56	S.5	100	S.4	” asymptomatic	74
M.J.	62	S.12	82	S.7	” ”	93
L.C.	45	S.15	99	S.16	” ”	98
A.B.	56	S.36	99	S.10	” ”	70
B.C.	62	S.37	80	S.34	” ”	92
H.R.	65	S.61	88	S.13	” ”	80
B.H.	44	S.62	87	S.14	Meningovascular syphilis	99
C.K.	60	B.C.	95	S.33	” ”	81
J.S.	62	C.H.	96	S.35	” ”	84
E.C.	69			S.38	” ”	82
J.M.	60			S.8	Neurosyphilis with optic atrophy	86
H.C.	57			S.9	E.R.B. spastic paraplegia	85
L.B.	68			S.43	Syphilitic muscular atrophy	81
G.C.	64					
M.D.	48					
Avg index of growth in 2% sol.	58.5%		91%			84.5%

low those given by normal spinal fluids, the average phytotoxic index being 58.5%. In fact the spinal fluids were relatively more toxic than the blood sera of the same patients. On the other hand, the 10 specimens of spinal fluids from locomotor ataxia, gave an average reading of 91%, practically normal. The series of other neuro-syphilitic cases also gave quite high readings, the average being 84.5%.

All of the above cases were, of course, of syphilitic origin and gave positive Wassermann tests. Fortunately, however, the author has already pointed out elsewhere that syphilitic blood sera when tested on *normal* seedlings, produce no toxic inhibition and usually give even slightly higher indices of growth(7). The phytotoxic properties of paretic spinal fluid therefore must be ascribed to biochemical changes inherent in the psychosis. This difference between paresis and other neuro-syphilitic patients may be of interest from the standpoint of localizing the seat of the psychotic affection.

Summary. 1. A phytopharmacological examination of spinal fluids from a series of paresis cases yielded marked phytotoxic results. 2. Spinal fluids from a series of patients with *Tabes dorsalis* gave the same readings as those of normal spinal fluid. 3. Other cases of neuro-syphilis also yielded almost normal indices of growth. 4. The above phytotoxic properties of paretic spinal fluids cannot be ascribed to syphilis because syphilitic blood sera and spinal fluids, alone, from non-psychotic patients are not phytotoxic.

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Difference in Intestinal Absorption Between Vitamin A Alcohol and Ester.* (17621)

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Vitamin A is transported from the intestinal tract to the liver in ester form and is stored as such in the liver(1). It is presumed to pass through the intestine wall as the free alcohol even if given in esterified form. However, it has been observed in normal children that the administration of free vitamin A results often in lower blood levels than that of an equal dose of the esters(2). The contrary was seen in the newborn or in children with pancreatic diseases or with other pathologic conditions which do not necessarily influence intestinal absorption(2,3,4). Recently(5), fluorescence microscopic studies of the intestinal absorption of vitamin A in rats revealed that vitamin A alcohol is less efficiently absorbed than are the vitamin A esters. It appeared, therefore, pertinent to compare the absorption of free vitamin A with that of vitamin A esters as measured by the response of the plasma vitamin A level to a test dose of vitamin A. This was done in a group of adult patients including cases of liver diseases in which the intestinal vitamin A absorption is known to be impaired. It appeared also interesting to study the influence of dispersion upon both vitamin A preparations and furthermore to establish what percentage of vitamin A is esterified in the blood after administration of any of the different preparations used.

Material and Method. The blood vitamin A level of 16 hospital controls and 14 patients with liver diseases including acute hepatitis, cirrhosis and obstructive jaundice was determined according to the method of Kimble(6) (using the Coleman spectrophotometer) after an intake of a test dose of 75,000 units of vitamin A unless otherwise specified. In 4 cases, in addition, the vitamin A esters were determined according to a slight modification(7) of the procedure of Gillam and Senior(8). The alcohol vitamin A was calculated by subtraction of the vitamin A esters from the total vitamin A level. Vitamin A was given (usually with some lemon juice) in preparations containing 25,000 units per cc either as the alcohol in the form of a vitamin concentrate (unsaponifiable fraction of fish liver oil) or as an especially prepared vitamin A palmitate or acetate. Both preparations were either dissolved in corn oil or dispersed in water with an appropriate nonionic emulsifier (fatty acid polyoxyalkylene derivative).† In an additional series, the amount of vitamin A administered was slightly varied.

Results. In hospital controls, equal amounts of vitamin A palmitate in aqueous menstruum raised the blood vitamin A level slightly more than those of vitamin A alcohol. This difference was not marked in all instances and appeared reversed in 2 out of 16. Similarly, vitamin A palmitate in oily solution produced, as a rule, a more marked response of the plasma vitamin A than vitamin A alcohol (Fig. 1). On the average, vitamin A alcohol in aqueous menstruum produced approximate-

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* Supported by a grant from Endo Products, Inc.

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† The vitamin A preparations were provided by Dr. Samuel M. Gordon, Endo Products, Inc.

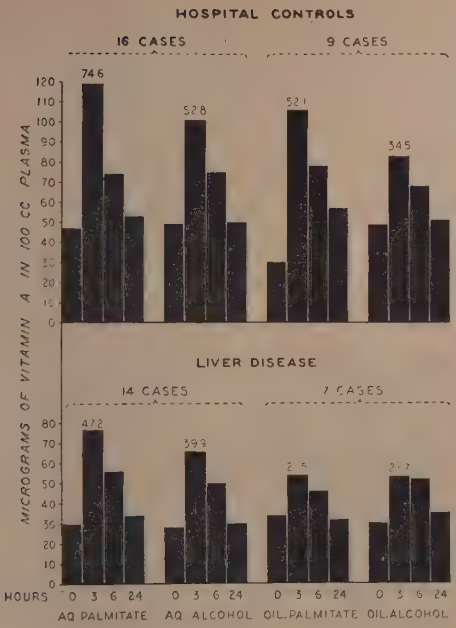

FIG. 1.

Average of plasma vitamin A level before, 3, 6, and 24 hours after administration of 75,000 units of vitamin A as palmitate or as alcohol in aqueous and oily menstruum in hospital controls and patients with liver diseases. The figure over the highest bar indicates the average maximal difference between the plasma vitamin A level before and after intake of the test dose of vitamin A.

ly the same rise of the plasma vitamin A level as vitamin A palmitate in oily solution. None of the differences, however, were statistically significant. Thus, the T value of the difference between aqueous emulsion of vitamin A alcohol and palmitate was 1.8, the lower limit of statistical significance being 2.5. In patients with liver diseases, an almost similar behaviour was noted except that the response of the plasma vitamin A level to the same dose of vitamin A was significantly lower than in the hospital controls. This is more apparent with the oily preparations which hardly produced any difference in plasma levels of vitamin A after ingestion of vitamin A alcohol or palmitate.

The plasma vitamin A level of 6 patients who received vitamin A palmitate in significantly smaller amounts than vitamin A acetate or alcohol (all in aqueous emulsion) rose, on the average, almost equally; it rose more after acetate given in the same amount as the alcohol (Table I). In the two controls and the two patients with hepatic cirrhosis in whom the plasma vitamin A was partitioned into alcohol and esters, the rise following the intake of alcohol or palmitate in both aqueous or oily menstruum was almost entirely due to an elevation of the esters (Table II).

Discussion. The present study indicates that in normal adults as well as in patients with liver diseases esterified vitamin A is not only as well absorbed by the intestine as the free vitamin A but slightly better. The difference in the absorption of the two forms however is not statistically significant. Superiority of vitamin A ester to alcohol(9,10) or of alpha tocopherol ester to free alpha tocopherol(10) in bioassays has been reported. If vitamin A passes the intestinal tract only in the free form, the necessary lipolytic process does not seem to retard the absorption of esterified vitamin A. Vitamin A is transported in the blood from the intestinal tract to the liver in the ester form. If at least some vitamin A passes the intestinal wall in the esterified form (not requiring subsequent esterification in the intestinal wall) the trend for better absorption of the esterified form would be explained. However, it has been shown that in pancreatic disorders the free form is better absorbed and it has even been claimed that a characteristic difference between celiac disease and pancreatic disorders lies in the poor absorption of the esters in the latter(2). In hepatic diseases in which the

TABLE I.
Average Maximal Difference Between Plasma Vitamin A Level Before and After Intake of Various Doses of Vitamin A Alcohol, Palmitate, and Acetate in Aqueous Emulsion in 6 Patients.

Form of vit. A admin.	Amt in units	Avg max. rise of plasma vit. A level in μm per 100 cc
Alcohol	82,500	56.5
Palmitate	69,300	61.8
Acetate	83,800	73.3

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TABLE II.
Mean Plasma Levels of Total and Ester Vitamin A in 2 Normals and 2 Patients with Hepatic Cirrhosis Before and at the Peak After Administration of 75,000 Units of Vitamin A as Palmitate or as Alcohol in Aqueous or Oily Menstruum.

	Mean Plasma vitamin A in μm per 100 cc in two							
	Controls				Patients with cirrhosis			
	Prevalue		Peak		Prevalue		Peak	
Vit. A preparation administered	Total	Esters	Total	Esters	Total	Esters	Total	Esters
Aqueous palmitate	44.2	13.8	120.1	79.3	15.2	7.3	44.0	32.0
” alcohol	46.0	8.7	89.9	42.7	9.6	3.3	23.9	14.2
Oily palmitate	44.9	11.4	66.8	47.6	8.0	3.8	22.4	15.9
” alcohol	44.7	4.9	57.7	21.9	7.7	3.7	13.5	3.4

deficiency of bile acids usually impairs lipolysis, this difference does not exist. The findings can be explained either by a better stability of the esters protecting them from oxidative destruction in the intestine(10) or by the assumption that vitamin A is absorbed partially as alcohol and partially as esters. While in normals the esters are slightly better absorbed, in pancreatic disorders the defect in lipolysis outweighs this advantage. Against the hypothesis that the higher blood levels following the administration of the esters are the result of a delayed uptake by the liver speaks the fact that the absorbed vitamin A in the blood stream is in the ester form independent of the form it was originally fed(7). Moreover, the microscopically observed better absorption of vitamin A in the gut of animals following administration of the esters does not support that hypothesis. From a practical point of view, the state of esterification appears of less significance for the absorption of vitamin A, despite the slight advantage of the ester, than the degree of dispersion which is equally important for the absorption of ester as of alcohol. Recently, ample evidence has been presented for the better absorption of aqueous preparations of vitamin A in comparison to the oily ones(11,12,5).

Summary. The plasma vitamin A level of hospital controls and patients with liver diseases was, as a rule, slightly more elevated after the intake of equal doses of vitamin A esters than of the alcohol. This difference was noted if the preparations were given in oily as well as in aqueous menstruum. Equal amounts of vitamin A in aqueous medium produced regularly a higher plasma level than in oil. The rise of the plasma level was mainly due to elevation of vitamin A esters whether vitamin A was administered as alcohol or as ester. Lipolytic processes, therefore, do not retard the absorption of vitamin A and it is suggested that part of vitamin A may be absorbed as ester. The greater stability of the esters may be an alternative explanation.

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Electron Microscopy of Embryonic Avian Tissues.* (17622)

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Although tissues thin enough for visualization by electron microscopy may be obtained by tissue culture(1), by sectioning(2,3) and by mounting single cells(4), the methods available are time-consuming, and require considerable skill and experience. The present report will describe a method that appears relatively easy and satisfactory for obtaining preparations of embryonic avian tissue suitable for electron microscopy. The principle of the method depends upon obtaining growth of tissue directly upon the wire mesh discs or grids on which specimens conventionally are mounted with the aid of a collodion or plastic membrane. This may be accomplished by inserting the discs into the chorio-allantoic cavity of developing chick embryos. After a day or two the discs appear to become attached to the embryonic membranes and cells grow into the meshes, attaching themselves to the edges of the wire strands.

Materials and Methods. Wire mesh discs of the type used in mounting specimens for electron microscopy were sterilized in boiling water and, without further treatment, were inserted directly into the chorio-allantoic cavity of embryonated hens' eggs which had been incubated at 37°C for 6 to 11 days. Taking care to avoid tearing the chorio-allantoic and shell membranes, a piece of shell about 0.5 cm in diameter was removed from the area overlying the chorio-allantoic membrane, in the vicinity of the embryo. A disc was then grasped with a pair of fine forceps and inserted into the chorio-allantoic cavity by directing the edge of the disc parallel to the fibers of the shell membrane and quickly forcing it through the membranes. This procedure produced a small slit in the membranes through which additional discs could be inserted. As a rule, 3 or 4 discs were placed in each egg. The exposed area was then covered with a small piece of scotch tape and the eggs replaced in the incubator. After 3 to 5 days the eggs were candled to determine viability and, in some instances, sterile phosphate buffer or normal allantoic fluid was inoculated into the chorio-allantoic sac. Not infrequently the air sac was found to have shifted to the side, and when material was injected subsequent to the insertion of discs care had to be taken to insure that the chorio-allantoic sac had been entered. Candling, however, usually revealed an area of chorio-allantoic membrane, adjacent to the newly formed air sac, through which inoculation was carried out. A small hole was drilled through the shell over this area and inoculation was done in the manner usually employed. Eggs which had been inoculated were further incubated at 37°C for several hours. Samples of allantoic fluid were then removed aseptically; the egg shells were broken open, and the contents poured into Petri dishes. The discs were removed and placed in distilled water where they were teased free of visible bits of tissue and washed for a minute or two. Discs were placed on a paper towel to remove excess water and finally allowed to dry at room temperature for 5 to 10 minutes. Preparations which appeared grossly unsuitable for electron microscopy on preliminary examination under low magnification with a light microscope were discarded. Specimens considered unsuitable

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* Aided by grants from the U. S. Public Health Service, the Commission on Influenza, Army Epidemiological Board, and the Medical Fluid Research Fund of the Yale University School of Medicine.

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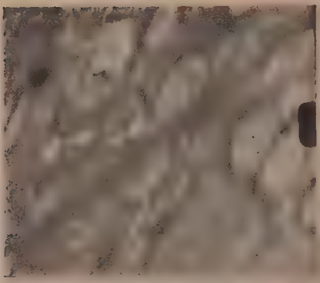

Fig. 1

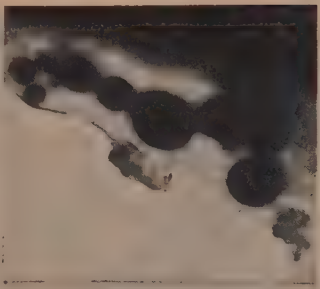

Fig. 2

Fig. 3

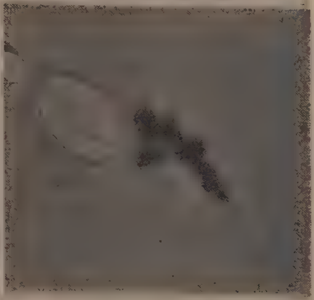

Fig. 4

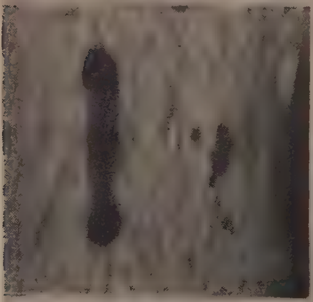

Fig. 5

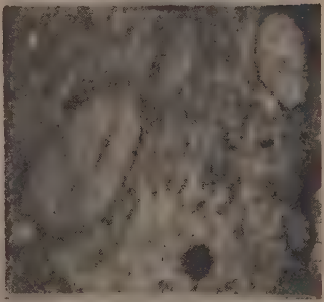

Fig. 6

FIG. 1. Cytoplasm from 10-day embryo; grids in place 4 days.
FIG. 2. Cytoplasm from 11-day embryo; grids in place 3 days.
FIG. 3. Cytoplasm from 11-day embryo; grids in place 5 days.
FIG. 4. Cytoplasm from 11-day embryo; grids in place 5 days.
FIG. 5. Cytoplasm from 11-day embryo; grids in place 4 days.
FIG. 6. Different portion of same preparation shown in Fig. 5.
Magnification × 13,400 except Fig. 1, which is × 8400.

Preparations shown in Fig. 1-4 were not fixed; that shown in Fig. 5 and 6 was fixed by exposure to osmium tetroxide for 30 minutes.

were those in which the layer of tissue was obviously too thick as well as those in which there was no tissue within the small central zone of the disc which could be visualized under the electron microscope. Most preparations were then examined, without further treatment, in an RCA electron microscope, type EMU. All fresh specimens were examined within one to four hours after removal of the discs. A few preparations were fixed by exposing them to fumes of 2% osmium tetroxide for a period of 15 to 30 minutes.

Cultures for bacterial contaminants were done, aerobically only, by plating allantoic fluid on blood agar and incubating at 37°C.

Experimental. Although no attempt has been made, as yet, to identify types of tissue or to visualize and reconstruct pictorially single cells as such, a great variety of cellular detail has been observed and studied. Selected preparations illustrating, in part, the variety of minute detail which has been observed in cytoplasm, are depicted in the photographs reproduced in Fig. 1-6. Much variation has been encountered in different specimens, even in individual discs from the same egg. A number of structures previously described, such as mitochondria and Golgi bodies(1), have been recognized. In addition, many other less clearly defined structures have been seen.

Discussion. Experience derived from examination of tissues from about 50 embryos suggests that avian tissues suitable for electron microscopy may be obtained by the method described. Although most of the discs prepared in this way contain a considerable amount of tissue in the peripheral areas, many contain little or no tissue in the central areas. The fact that only the small central zone of a disc may be visualized under the electron microscope thus renders many preparations unsuitable for study. Furthermore, even when sufficient tissue is present in the central area, in some instances, it is too thick for visualization. However, when several discs are inserted into each egg usually at least one contains some tissue which may be visualized. Since the tissue supports itself, apparently by attachment to the wire strands of the discs, a supporting membrane of collodion or plastic is not required. This fact allows great contrast in portions of very thin tissue and as well perhaps allows reasonably good visualization of tissues thicker than those which could be visualized satisfactorily if a supporting membrane were present. Satisfactory specimens have been obtained in various circumstances but optimal conditions as regards age of embryo and time of removal of discs have not been established.

Mortality following insertion of the discs has varied considerably but appears to average about 34%. Bacterial contamination of the allantoic cavity has occurred in only 8% of the embryos examined. Application of the method described to the study of embryonic avian tissues infected with various agents and to the study of both normal and abnormal tissues of other species is planned.

Summary. An easy and satisfactory method of obtaining embryonic avian tissues for electron microscopy has been described. A variety of detailed cellular structure has been observed and illustrated.

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Effects of Androgen and Somatotrophin on the Os Penis of the Rat.* (17623)

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In a previous paper(1) it was reported that testosterone propionate acted as a growth stimulant to the rat's os penis in the absence of the pituitary gland. There remained the question of what influence somatotrophin (growth hormone) might have upon the penile ossicles. Would it be capable of stimulating bone growth in a penis that remains atrophic and poorly vascularized in the absence of androgens? Or would it act with an androgen to bring about a better growth than the androgen alone could accomplish? The following

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* Aided by grants from the Research Board of the University of California.

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TABLE I.
Showing the Effect of Testosterone Propionate and Somatotrophin on Body Weight and Os Penis of Castrated, Hypophysectomized Rats.

No. of rats	Operations*	Testosterone propionate, mg	Somatotrophin, mg	Avg wt		Avg wt gain, g	Avg area of lateral radiophic shadow of os penis, mm^2
				Day 26	Day 47		
5	C & H	0	0	74.8	83.4	8.6	1.28 ± .08
5	C & H	0.1	0	72.2	86.6	14.4	2.20 ± .10
5	C & H	0	1.0	75.8	145.8	70.0	1.50 ± .02
5	C & H	0.1	1.0	79.0	165.2	86.2	2.78 ± .05
4	C	0	0	68.5	156.5	88.0	1.38 ± .14
5	26 day normal	0	0	70.4	—	—	1.35 ± .07
5	47 ” ”	0	0	—	168.8	—	2.29 ± .19

* C—castration; H—hypophysectomy.

experiments have contributed answers to these questions.

Experimental. In this investigation the os penis was studied histologically and planimetrically in the following groups of Long-Evans rats: (1) castrated and hypophysectomized when 26 days old and injected subcutaneously on the same day and daily thereafter for 21 days with 0.1 cc sesame oil; (2) similarly operated upon and injected daily subcutaneously with 0.1 mg testosterone propionate[†] in 0.1 cc sesame oil for 21 days; (3) similarly operated upon and injected daily subcutaneously with 1.0 mg of somatotrophin[†] (pituitary growth hormone) for 21 days; (4) similarly operated upon and injected daily subcutaneously with 0.1 mg testosterone propionate and 1.0 mg of somatotrophin for 21 days; (5) castrated only when 26 days old and received no hormone; (6) 26-day-old normal rats; (7) 47-day-old normal rats. All rats were fed the customary dry diet and water *ad lib.* as well as a wet diet made up in the form of a paste especially for the benefit of the hypophysectomized animals. On the day following the last injection, or at 47 days of age, the penis and the fore-paws were fixed in 10% formalin and radiographs made of these tissues immediately thereafter. Radiographs were also made of the whole rat in some cases. Instead of comparing the penile ossicles in the different groups by merely measuring their lengths microscopically as in the previous report(1), planimetric studies were made on enlarged skiagrams (prints enlarged 3.5 X from contact films made of the lateral aspect of the distal part of the penis). Histologic studies were made on the penis and the third metacarpal; but the metacarpals will not be treated in this report except to say that they were used as control bones providing the expected evidence of an excellent growth response to somatotrophin in contrast to the os penis.

Results. In Table I, the results obtained in the various groups are summarized. The earlier findings of a slight weight increment (8.6 g in 21 days) in hypophysectomized rats of this age uninjected with hormone and the slightly greater increment (14.4 g) in those receiving 0.1 mg daily of testosterone propionate was confirmed. However such rats remained stunted in their growth when compared with normal animals or those receiving somatotrophin (see skiagrams in Plate 1). The rats injected with a daily dose of 1.0 mg of somatotrophin gained 70 g in the 21-day period as contrasted with those receiving 1.0 mg of somatotrophin and 0.1 mg of testosterone propionate which gained 86.2 g. This latter figure falls within the range of weight increments observed in normal rats during this period and happens to approximate the sum of the increments observed in the groups injected separately with somatotrophin or testosterone (70 plus 14.4).

The figures in the right hand column of Table I represent the area of the lateral aspect

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[†] The somatotrophin was generously provided by Dr. C. H. Li, Institute of Experimental Biology, and the testosterone propionate by Ciba Pharmaceutical Products, Inc., Summit, N. J.

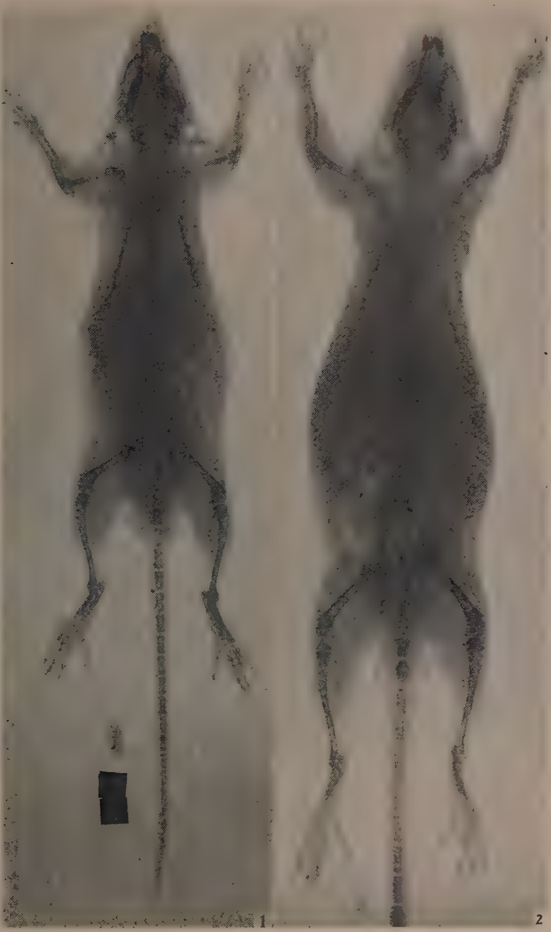

PLATE 1.

FIG. 1. Skiagram of a rat castrated and hypophysectomized at the age of 26 days and injected subcutaneously daily for 21 days with 0.1 mg testosterone propionate. × 0.5.

FIG. 2. Skiagram of a rat castrated and hypophysectomized at the age of 26 days and injected subcutaneously daily for 21 days with 1.0 mg somatotrophin. × 0.5. In each figure, the peripheral part of the penis containing its minute ossicle has been placed beside the tail. Enlarged skiagrams of os penis may be seen in the following plate. In the rat shown in Fig. 1 the skeleton remained stunted and the os penis and the penis grew, whereas in the other rat normal skeletal size was attained but the os and the penis remained small.

of the os penis as projected against a contact X-ray film. The enlarged skiagrams were measured with a planimeter and corrections then made for the enlargement. The figures of 1.28 and 1.38 mm^2 for the hypophysectomized and castrated, and the simply castrated animals respectively indicate that a growth stasis occurred in the os penis in both of these groups (Fig. 7 and 11, Plate 2). The figure obtained for the 26-day-old normal rat was

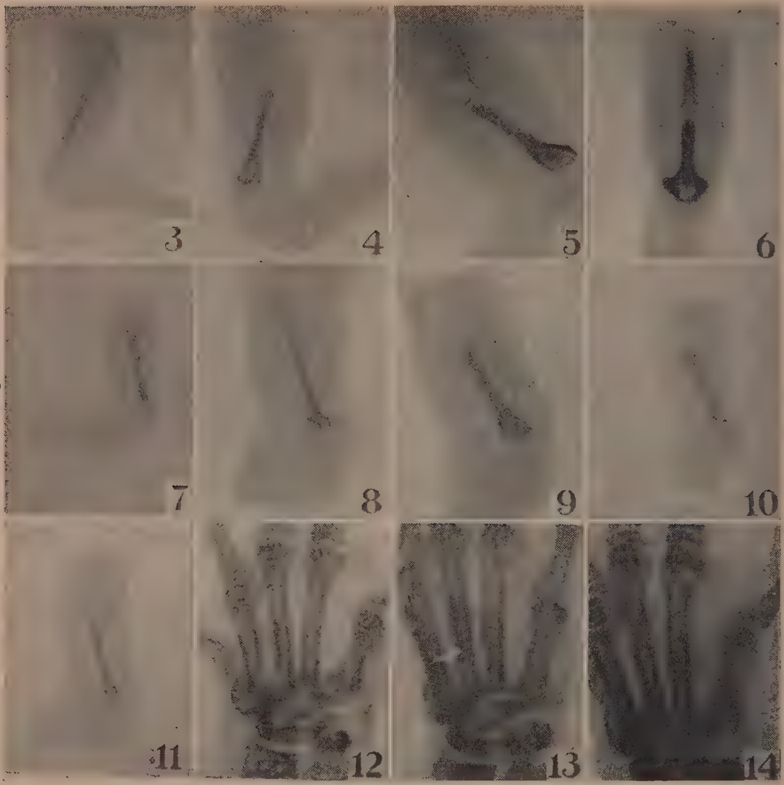

PLATE 2.

FIGS. 3-12. Skiagrams (all lateral views except Fig. 6 which is antero-posterior) of penile ossicles of rats under the following conditions or treatments. All figs. 3.5 ×.

FIG. 3. 26 days old normal.

FIG. 4. 47 days old normal.

FIG. 5. 3 months old normal showing secondary ossicle that forms in the glans penis distal to the main bone between the second and third months.

FIG. 6. Same in A-P view.

FIG. 7. Castrated and hypophysectomized on day 26 and injected daily subcutaneously with sesame oil for 21 days. Necropsy day 47.

FIG. 8. Similarly operated and injected daily subcutaneously with 0.1 mg testosterone propionate in sesame oil for 21 days. Necropsy day 47. This ossicle is almost identical in size and shape with that of the normal 47-day-old rat (Fig. 4).

FIG. 9. Similarly operated and injected as in the preceding case plus a daily subcutaneous dose of 1.0 mg somatotrophin for 21 days. Necropsy day 47. The area represented here is 20% greater than that of the preceding case receiving androgen alone. Most of the overgrowth is at the spatula-like proximal cartilaginous end.

FIG. 10. Similarly operated, but received only somatotrophin. Same regimen as in preceding case.

FIG. 11. Castrated only, day 26. Necropsy day 47.

FIGS. 12, 13, 14. Skiagrams of forepaws of rats shown in Figs. 8, 9, and 10, respectively, for comparison of the middle metacarpals (3rd from right in each case) illustrating the main finding, namely, that the rats receiving androgen showed good growth of the os penis (Figs. 8 and 9), but not of the general skeleton (Fig. 12), whereas the rats receiving somatotrophin showed good growth of the general skeleton (Figs. 13 and 14), but not of the os penis (Fig. 10). All × 3.5.

1.35 mm^2 (Fig. 3, Plate II). The doubly operated animals injected with somatotrophin alone showed only a very slight increase in size of the os penis over that of the normal 26-day-old controls (Fig. 10, Plate 2), the average area being 1.5 mm^2. Testosterone propionate caused a growth of the os penis of doubly operated rats (Fig. 8, Plate 2) comparing favorably with that of normal controls (Fig. 4, Plate 2) at the age of 47 days (2.20 mm^2 as compared to 2.29 mm^2). The best growth of this ossicle (2.78 mm^2) was obtained in the doubly operated group injected with both somatotrophin and testosterone propionate (Fig. 9, Plate 2). Here, as in the case of the body weight increments, there seemed to be an additive effect (or synergism) of the 2 hormones. In no case did the amounts of hormones used bring about full maturation of the main ossicle or the ossification of the distal center. These processes are only accomplished by about the third month (see Fig. 5 and 6, Plate 2). In contrast to its negligible effect on the os penis, somatotrophin caused excellent growth of the general skeletal system (Fig. 1 and 2, Plate 1, and Figs. 13 and 14, Plate 2).

The bones of the fore-paws of the doubly operated rats receiving testosterone propionate alone were essentially similar to those of the doubly operated controls injected with sesame oil.

Histologic studies revealed that the sections of the os penis from castrated and hypophysectomized rats injected with somatotrophin could not readily be distinguised from the doubly-operated controls given sesame oil. In both groups an occasional small cluster of cartilage cells had persisted in some rats for the 21-day period; but in all there appeared to be a lack of the stimulus for chondrogenic mesenchyme to transform to cartilage as well as a cessation of cartilage cell growth and bone replacement. The rats castrated only, showed slightly less regression of the cartilaginous proximal cap by the 47th day; but here also, the bone remained atrophic as in the doubly operated controls. In the rats treated with testosterone propionate the main penile ossicles showed the same growth processes as were illustrated in the earlier report(1). New growth and hypertrophy of cartilage cells had kept pace with the chondrolysis that accompanied osteoblastic proliferation and ossification. Periosteal osteogenesis was comparable to that in normal animals of the same age.

This was also true of the rats treated with both hormones, but in these the ossicles were quantitatively better, as though slightly older —a finding apparently similar to that of Bok(2).

Summary. In contrast to its excellent effect in stimulating cartilaginous and osseous growth in the rat's penile ossicle, testosterone propionate, in a daily subcutaneous dose of 0.1 mg for 21 days following hypophysectomy and castration, caused no appreciable resumption of general skeletal growth. Somatotrophin (hypophyseal growth hormone) in a daily subcutaneous dose of 1.0 mg for the same period in similar animals caused excellent general skeletal growth, but did not correct appreciably the atrophic condition of the os penis. The two hormones synergized in causing better growth of the os penis than the androgen alone and a greater body weight increase than the growth hormone alone in doubly operated animals.

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Quantitative Aspects of the Protective Action of Cysteine Against X-Radiation. (17624)

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We have previously reported(1,2) that cysteine administration either orally or intravenously prior to the exposure of rats to lethal dosages of X-radiation greatly increases their chances for survival. As part of a series of studies pointed toward the characterization of this action of cysteine, experiments were designed for the purpose of revealing whatever quantitative relationships exist between cysteine dosage, X-ray dosage, and the level of the protective action of cysteine. The results of such experiments are presented in this paper.

Methods. In general the methods were similar to those reported previously(1). Control and experimental animals were irradiated simultaneously, caged together and otherwise similarly handled. Male Sprague-Dawley rats weighing 150 to 250 g were employed. Cysteine was administered as a 10% solution of the hydrochloride neutralized to pH 7-8 with 2 N sodium hydroxide. The material was injected into a tail vein within 5 minutes prior to irradiation. Animals were irradiated in pairs, one having been injected with cysteine and the other with an equivalent volume of 5 per cent sodium chloride. The irradiation consisted of a single total-body exposure to X-rays (250 kv, 15 ma, and 0.5-mm Cu and 3.0 mm Bakelite filters, 26.5 cm target distance, 1.5 mm Cu half-value layer and 210 to 220 r per minute dose rate). Survival at 30 days post irradiation was the chief criterion employed to evaluate cysteine protection. In addition, however, time to 50% mortality and the average survival time of animals dying within the 30-day post irradiation period were noted. Three types of experiments were performed: 1) the relative protective action of various dosages of cysteine (50 mg per kg to 950 mg per kg) was determined for given X-ray dosages (800 r and 1000 r); 2) the protective action of a given dosage of cysteine (950 mg per kg) was evaluated at various X-ray dosages (600 r to 1800 r); 3) since 950 mg per kg was found to be the maximum dose of cysteine tolerated by rats, the protection afforded by a dual injection whereby the total dosage of cysteine was increased, was evaluated for an X-ray dose of 1200 r. The dual injection consisted of a dose of 475 mg per kg followed in 45 to 60 minutes by a second dose of 950 mg per kg. The rats were irradiated immediately after the second injection (15 controls, 18 cysteine treated).

Results. The degree of protection afforded by cysteine against a particular dosage of X-radiation is roughly proportional to the cysteine dose (Fig. 1)

The degree of protection afforded by a given dosage of cysteine decreases as the X-ray dosage is increased (Fig. 2). A plot of the

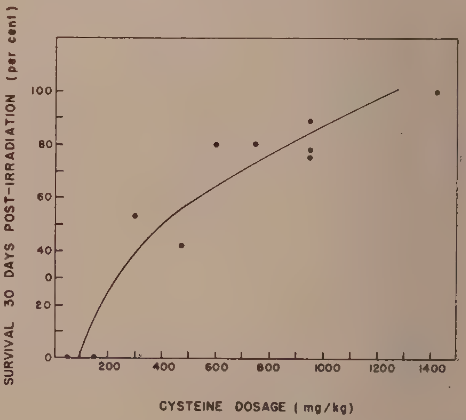

Fig. 1.

The influence of cysteine dosage upon the survival of rats exposed to total-body X irradiation (1000 r). Each point represents 9 to 18 rats. The 1425 mg/kg of cysteine point represents the dual injection experiment.

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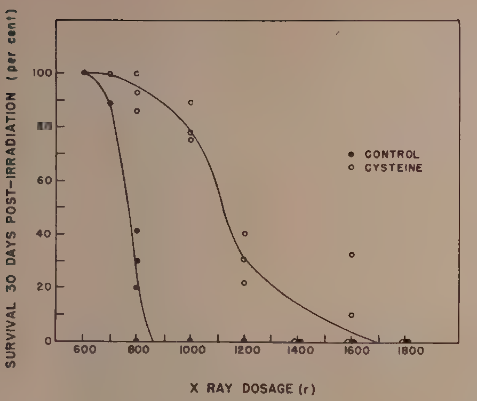

FIG. 2. The influence of X-ray dosage upon the survival of rats pretreated with cysteine. Each point represents 6 to 18 rats.

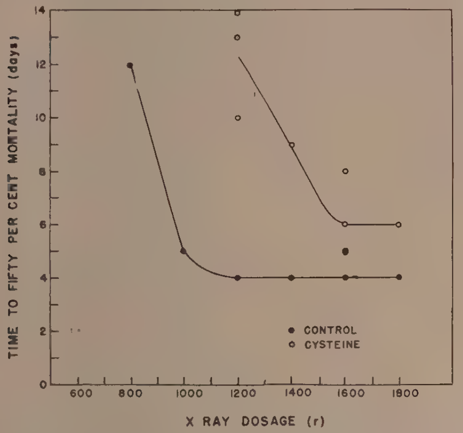

FIG. 3. The influence of X-ray dosage on time to 50% mortality of rats pretreated with cysteine. Each point represents 6 to 17 rats.

data upon logarithmic-probit graph paper yields straight lines.

A cysteine dosage of 950 mg per kg raises the LD_{50} from about 765 r to about 1120 r and almost doubles the LD_{100} (Fig. 2).

Cysteine increases the time to 50% mortality (Fig. 3). The average survival time of animals dying within 30 days post irradiation is also significantly increased.

Increasing the dose of cysteine 50% above the maximum tolerable single dose by means of dual injections 45 to 60 minutes apart protects 100% of animals exposed to 1200 r.

Discussion. The results of our experiments employing various cysteine doses at given levels of irradiation demonstrate the difficulties attending the evaluation of the effectiveness of substances which might alter radiosensitivity. Although exposure to 800 r in this laboratory usually kills about 80% of rats within 30 days, the range of mortality is from 60 to 100%. Only a rough indication of the relationship between cysteine dosage and protective action is obtained when the 30-day % survival is taken as an expression of cysteine protection at 800 r. The difficulties presented by the variation in control mortality after 800 r are somewhat lessened, however, and a better idea of the cysteine dose-cysteine protection relation is gained, if one considers the difference in per cent survival between control and treated animals which were irradiated simultaneously. Since an X-ray dose of 1000 r kills all control animals, it would seem from the above considerations and from the work of Clark and Uncapher(3) that testing at this level would allow one a better chance to assess the ability of materials to alter sensitivity to irradiation. The results of our experiments bear out this premise.

A more concise picture of the ability of cysteine to alter sensitivity to irradiation was gained from the series of experiments where the protection afforded by a given dosage of cysteine was evaluated for various X-ray dosages. Fig. 2 clearly demonstrates that the protective power of a given cysteine dose decreases as the amount of radiation is increased. When in addition to the data discussed above one considers the results of the dual injection experiment (100% survival at 30 days post 1200 r) it is clear that the level of protection afforded by cysteine against X-radiation is dependent upon cysteine dosage and radiation dosage.

The practicality of employing time to 50% mortality and average survival time as criteria for assessing the effectiveness of procedures which might alter radiosensitivity is well demonstrated in the present experiments. It is clear that cysteine can prolong life in instances where it does not save the animal from death.

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Summary. Experiments in which cysteine and X-ray dosage were varied over a wide range revealed that the protective action afforded by cysteine is proportional to the dosages of cysteine and radiation employed and that cysteine increases the time to 50% mortality and the average survival time of animals dying within 30 days post irradiation.

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Bacterial Inhibition by a Penicillin Moiety.* (17625)

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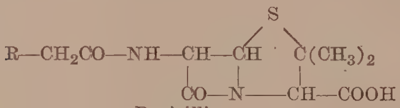
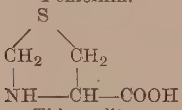
Little is known concerning the exact mode of action of most natural antibiotics, other than that they produce several general changes in bacterial morphology and respiration. Since biological activity can often be referred to specific groups in the molecule, metabolic studies involving smaller moieties of antibiotic molecules might possibly provide an approach to this problem. A preliminary attempt along these lines involving the nucleus of the penicillin molecule is described in this paper.

Experimental. 4-Thiazolidinecarboxylic acid, thioproline, was synthesized by the method of Armstrong and du Vigneaud(1). The organism, medium, and technics employed were those previously described and extensively used in analogue-metabolite inhibition studies(2). The results are summarized in Table I. Thioproline effectively inhibits the growth of *Escherichia coli* in mineral salts-glucose medium, and the inhibition is reversed to a limited extent by proline, despite the fact that the inhibitor is not isosteric with proline. To a somewhat lesser extent, a large number of other amino acids can also reverse the inhibition. Vitamins, purines, pyrimidines, and increased levels of glucose, however, are inactive in this regard. Combinations of amino acids manifest an even greater reversing ability than any single substance.



Penicillin.



Thioproline.

Discussion. A consideration of the data suggests that thioproline may inhibit bacterial growth by preventing the conversion of a large number of amino acids to some product or products essential for growth. It is apparently most effective in preventing proline utilization, perhaps because of its structural similarity to this amino acid. The known facts regarding penicillin action are not out of line with the possibility that penicillin prevents bacterial protein synthesis.

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* Part of this work was conducted under Dr. William Shive at the University of Texas in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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TABLE I.
The Effect of Various Amino Acids in Reversing the Toxicity of Thioproline for *Escherichia coli*. Organism incubated 16 hr at 37°C.

			Galvanometer readings* Thioproline (μg per 10 ml)			
No.	Reversing agent	Mg per 10 ml medium	0	300	1000	3000
1.	Blank	—	52	53	3	3
2.	DL-isoleucine	1	51	50	8	3
3.	L-histidine	1	50	50	49	4
4.	DL-leucine	1	52	51	53	5
5.	DL-valine	1	51	53	52	3
6.	DL-cystine	1	50	49	42	3
7.	DL-hydroxyproline	1	52	50	50	4
8.	DL-methionine	0.1	51	49	25	3
9.	L-aspartic acid	1	53	50	52	2
10.	L-glutamic acid	1	52	51	53	3
11.	L-lysine	1	49	50	51	3
12.	L-proline	1	50	52	53	12
13.	Glycine	1	54	50	17	3
14.	DL-alanine	1	51	49	49	3
15.	L-arginine	1	52	49	49	3
16.	DL-serine	1	54	49	3	3
17.	DL-threonine	1	51	52	3	3
18.	L-tyrosine	1	50	51	7	3
19.	L-tryptophan	1	49	53	4	3
20.	DL-phenylalanine	1	51	50	46	3
21.	1-5 above	.05 of each	53	52	52	25
22.	6-12 ”	.05 ” ”	52	49	50	42
23.	13-17 ”	.05 ” ”	50	52	51	34
24.	18-20 ”	.05 ” ”	53	51	52	3
25.	1-12 ”	.05 ” ”	53	54	51	42
26.	1-5, 13-17 above	.05 ” ”	51	52	52	43
27.	6-17 above	.05 ” ”	50	50	51	40
28.	Proline and cystine	.05 ” ”	50	51	51	42

* A water blank gives a zero reading; an opaque object reads 100.

Prolongation of Penicillin Blood Level by Implantation of Procaine Penicillin Tablets.* (17626)

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Many attempts have been made to prolong the penicillin blood level. Numerous procedures have been used to prolong the levels of penicillin in the blood. The three methods most commonly employed have been: *a*) a mixture of calcium penicillin with peanut oil and beeswax(1), *b*) the blockade of the renal elimination by carinamide(2,3), and *c*) the parenteral administration of relatively insoluble penicillin, such as procaine penicillin(4,5, 6). It has recently been demonstrated that implantation of tablets of procaine penicillin

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* Aided by the Gildemeister Foundation.

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TABLE I.
Penicillin Blood Serum Level After the Implantation of Procaine-Penicillin Tablets, in Days.

	Intramuscular				Subcutaneous				
	B.S.	M.P.	R.V.	M.E.*	M.E.*	F.S.	R.C.	L.A.	J.F.
	1.9	1.83	1.45	1.9	1.79	1.93	1.92	1.025	3.645
Doses per kg body wt	31.9	42.8	24.3	28.1	30.0	38.2	22.3	20.4	81
No. of tablets implanted	2	2	1	2	2	2	2	1	3
Oxford U. of Penicillin in 1 cc of Blood Serum.									
Time after implantation									
4-6 hrs	1.7	—	.36	.72	.62	—	.30	.20	4.34
1 day	.43	—	.28	.82	.36	.48	.62	.20	.55
2	.68	—	.18	.20	.40	.41	.56	.25	1.10
3	.68	.40	.18	.31	.50	.36	—	.22	.90
4	.43	.32	.20	.28	.38	.33	.37	.20	.84
5	.38	.39	—	—	.36	—	.40	—	—
6	.35	.35	.26	.32	.48	.36	.36	.20	.62
7	.30	.27	.22	.37	.40	.31	.24	.28	.48
8	.28	—	.25	.25	.25	.33	.32	—‡	.40
9	.29	.33	.21	.19	.21	.40	—	.24	.40
10	.31	—‡	.10	.14	—	.32	.30	—	.45
11	.34	—	.04	.05	.27	.27	.40	—	—
12	.40	—	.00	.00	—	—	—	—	—
13	.35	.36§			.28	.06	—	.01	.55
14	.38				—‡	.00	—		—‡
15	.22				.18		—		—
16	.15				—		.21		—
17	—‡				—		—		.21
18	.19				.00		.15		—
20	.00								.25
21-23									.40
24-28									.00

* Not the same patient.
† Doses implanted in thousands of units.
‡ Patient discharged from the hospital.
§ Patient lived more than 60 km from the hospital.

into the subcutaneous tissue of rabbits prolongs the penicillin blood level for 7 days, and that there is no local reaction in the site of the implantation. When procaine penicillin is mixed with fat, the subcutaneous implantation prolongs the penicillin blood level for 11 days(7). Implantation of pure procaine-penicillin tablets in human beings was attempted according to these results.

This paper demonstrates that the subcutaneous or intramuscular implantation of pure procaine-penicillin tablets is innocuous: there is no local or general reaction, and the penicillin blood level is prolonged for several days. Procaine penicillin was made according to the usual technic(8) from crystalline G sodium penicillin ("Merck"). The material was compressed very hard into tablets having a cylindrical shape 10 mm in diameter and approximately 1 g in weight. The tablets were sterilized. The blood serum penicillin titrations were made according to the technic used in the Clinic Laboratory of our hospital(9) and the results are expressed in Oxford units. As the tablets were not very small, they were laid in the subcutaneous or muscular tissue of patients a slight distance away from the surgical wound. No local or general symptom was observed, and the post operative course was very smooth. The perfect tolerance of subcutaneous or muscular tissue to procaine-penicillin tablets must be emphasized.

In Table I the results are summarized. The tablet implanted subcutaneously or intramuscularly produces a very prolonged penicillin blood serum level. The quantity of blood

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penicillin depends on the dose used. A high penicillin blood level was obtained with 3,600,000 units of procaine penicillin. A blood serum penicillin level very superior to the minimum effective level was obtained with implantation of 1,000,000 to 2,000,000 units of procaine penicillin.

Summary. Tablets of procaine penicillin implanted in the subcutaneous or muscular tissue provide a safe procedure for prolonging the penicillin serum blood level for several days.

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Biochemical Studies on the Alligator. (17627)

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In the course of a search for a new experimental animal for certain biochemical studies it was decided to investigate the suitability of the large North American alligator, *A. mississipiensis*. Some preliminary analyses revealed a striking discrepancy between some of the results obtained in the present study and those reported earlier by Hopping(1), Dill and Edwards(2), and Austin *et al.*(3). Most of the work that had been done on the alligator was repeated by the present authors and many additional studies were conducted. This paper deals with the results obtained on the fasting alligator kept under laboratory conditions. The effect of feeding, temperature, and seasonal variation on the composition of the blood and urine is quite marked and will not be reported here.

This study includes the data obtained from 65 alligators of undetermined sex. Twelve were 3-4 years old and weighed from 1.20-7.37 kg; 5 were 2-3 years old and weighed 0.79-2.03 kg; and 48 were about one year old and weighed from 48-182 g. The animals were kept at an average temperature of about 26°C in monel metal cadaver tanks which were filled to a depth of about one inch with water. They were fed fresh skinned rats from our colony. Although the alligators were avid feeders in the early spring and summer they tapered off in October and November and ate very little in December, January, and February. Under these conditions the animals doubled their weight in a 12 month period.

Blood was withdrawn from the tip of the tail for glucose determinations. All other determinations were done on blood obtained conveniently by cardiac puncture by the method suggested by Hopping(1). Amounts up to 10 ml were drawn by this procedure without injury to the alligator. The electrolyte studies were conducted on blood kept under mineral oil using potassium or ammonium oxalate as the anticoagulant. Urine was obtained by cloacal catheterization using a number 14F catheter.

Analytical Methods. Ammonia and urea of the blood and urine were determined by the method of Van Slyke and Cullen(4), uric acid by the method of Folin(5) and Brown(6), creatinine by the method of Folin and Wu(7), and protein and N.P.N. by the micro-Kjeldahl method of Howe(8). The pH determinations were done on a Beckman Model G pH meter with the Beckman glass electrode designed for blood analyses. Chlorides were determined

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TABLE I.
Blood Studies.

	No. det'ns	Avg	Range
Cell count, mm^3	29	481000	331000-676000
White Cells, %	33	6.84	4.7-10.8
H'crit, %	29	21.8	16-29
M.C.V., μ^3	29	472	361-562
Sed. Rate, mm/hr	33	5	2-7
Hb., %	15	7.0	5.0-9.2

by the method of Schales and Schales(9) and the bicarbonates with the Van Slyke(10) manometric apparatus. The Beckman flame photometer was used to determine sodium, potassium, and magnesium. Serum calcium was done by the Clark-Collip(11) modification of the Kramer-Tisdall(12,13,14) method, urinary and fecal calcium by flame photometry, and serum phosphorus by the method of Kuttner and Cohen(15). Blood glucose was determined by the method of Folin and Wu(16) and liver glycogen by the method of Good *et al.*(17). Total blood volume was estimated by the use of Evans Blue dye(18). The Klett-Summerson Photoelectric colorimeter was used for the colorimetric determinations.

Results. Table I presents a summary of the results of the general blood studies which were conducted throughout the year. For some reason our values for hematocrit, cell count, and mean corpuscular volume are quite dissimilar from those reported by Hopping. Calculation of the M.C.V. from the data of Hopping gives a value of 160 cubic micra as compared with the 472 cubic micra we found. It is possible that the reported cell counts were inaccurate since the dimensions she reported for the individual cell are in keeping with our own observations. The blood volume was determined on one alligator weighing 2 kg and was found to be 4.2% of the body weight. No other blood volume determinations were conducted since the dye colored the blood for a period of many weeks which made it unfit for colorimetric determinations of its constituents.

Hundreds of glucose determinations were done at various times and at every month throughout the year. There was considerable seasonal variation in the results obtained. The "average" figure of 99 mg % includes individual values for about 25 alligators for each month of the year. For the 300 analyses reported here the highest fasting level found was 205 mg% (August) and the lowest was 21 mg% (November). Liver glycogen analyses were done on 2 animals; one had a glycogen content of 9.62% of the net weight of the liver and the other 8.00% (July).

The blood N.P.N. and ammonia determinations were made during the summer, fall, and winter months. The values obtained in this study (Tables II and III) are much higher than those previously reported(1,3). It is apparent that ammonia is not only present in the blood in considerable amounts in these alligators but is indeed the chief nitrogenous constituent of both blood and urine. It is improbable that any error was made in these determinations since ammonia was determined on all blood samples immediately after withdrawing blood and since the N.P.N. as determined by the Kjeldahl method was also much higher than that found by Hopping and Austin *et al.* for their alligators. The failure of Hopping to find

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TABLE II.
Blood Nitrogenous Constituents.

		No. det'ns	Avg	Range
Blood	NH_3, mg%	27	61	18-98
"	Urea "	10	0	—
"	Uric Acid, mg%	42	2.25	0.96-4.23
"	Creatinine "	27	1.80	0.40-3.12
Serum	NPN, mg%	22	90.8	57.4-132.8
"	Protein, %	14	5.02	3.37-6.02

TABLE III.
Urine Nitrogenous Constituents.

	No. det'ns	Avg	Range
NH_3, mg%	23	216	116-262
Urea, "	9	tr.?	0-tr.?
Uric Acid, mg%	20	101	30-208
Creatinine, "	19	3.5	2.2-6.1
Total N, "	6	273	208-334

TABLE IV.
Blood Electrolytes.

		No. det'ns	Avg	Range
Blood	pH	58	7.30	6.87-7.66
"	Cl, meq/L	18	96	80-107
"	K, "	4	32.8	23.1-44.9
Plasma	Na, "	29	154	146-162
"	K, "	10	4.2	2.6-6.4
"	HCO_3 "	18	21.9	14.3-39.3*
"	Cl, "	29	107	83-128
Serum	Ca, mg%	12	10.2	9.4-11.0
"	P, "	11	3.39	2.86-3.85

* One alligator maintained a persistent alkalosis over a period of several months. It is improbable that any of the other animals would ever approach a fasting HCO_3^- level of 39.3.

TABLE V.
Urine Electrolytes.

	No. det'ns	Avg	Range
pH	62	7.72	7.18-8.02
$\overline{HCO_3}$ meq/L	13	105	77.6-125
Cl, "	30	tr.	0-tr.
Na, "*	9	3.19	Pooled
K, "*	9	11.1	"
Ca, "*	9	13.3	"
Mg, "*	9	1.0	"

* Done on ashed specimens.

creatinine in the blood is difficult to explain. Although the urinary ammonia is fairly consistent at a level of about 0.1 M. we were unable to detect any measurable quantity of urea in the blood or urine.

Blood and urine electrolyte studies were conducted during the summer months and the results are summarized in Tables IV and V. The principal cation of the urine is NH_4^+ and the principal anion is $\overline{HCO_3}$. The kidney of the fasting alligator seems to conserve both sodium and chlorine to a considerable extent since neither of these ions was present in any quantity in the urine although both were present in the blood in amounts similar to those reported for man. The alligator is apparently exceptionally well adapted for conserving sodium chloride since he loses very little in the feces (Table VI).

The pH of the urine of the fasting alligator

TABLE VI.
Composition of Fecal Ash.

	Dry wt, g	Ash, g	Cl	CO_3, %	Na, %	K, %	Ca, %	Mg, %
Sample 1	14.42	9.38	tr.	51.47	.416	.037	30.92	.427
” 2	0.97	0.76	0	—	.215	.026	32.26	.827

TABLE VII.
Composition of Alligator Ash.

Alligator, No.	Wt, g	Dry wt, g	H_2O, %	Ash, g	Cl, mg	Na, mg	K, mg	Ca, mg	Mg, mg
1	71.6	12.9	81.9	2.459	42.25	112.5	130.2	290	16.4
2	91.5	20.7	77.4	3.001	120.20	155.0	161.0	565	9.3
3	68.0	14.5	78.7	2.319	89.25	106.6	111.9	327	18.8

is always more alkaline than that of the blood. The blood pH shows a remarkable variation from animal to animal and even in the same animal at different times of the day. The resting pH is very difficult to determine since hyperventilation occurs when the animal is excited, and consequently it was necessary to do a large number of determinations on blood and urine because of the great variations found. Analyses of 2 specimens of fecal ash of alligators on a diet of fresh skinned rats are reported in Table VI. The dried feces are gray in color and contain almost no organic matter. The principal component seems to be $Ca(HCO_3)_2$. Three small alligators were also ashed in an electric furnace and the results are recorded in Table VII.

Comments. Neither Hopping(1), Dill and Edwards(2), nor Austin *et al.*(3), used alligators which were known to have fasted before their studies were made. It should be emphasized here that all chloride, bicarbonate, pH, and sodium determinations must be conducted on alligators which have fasted for at least 3 days because of the great magnitude of the "alkaline tide" with all of its accompanying changes in blood electrolytes(19).

It has been our experience that alligators make excellent laboratory animals for many types of experiments. They are hardy, easy to feed, require much less laboratory space than mammalian forms of comparable size, and, in spite of popular opinion to the contrary, they are easy to handle.

Summary. The following determinations have been done on fasting alligators: blood cell count, % white cells, hematocrit, M.C.V., sedimentation rate, hemoglobin, blood vol., blood glucose, liver glycogen, blood and urine ammonia, urea, uric acid, creatinine, serum proteins and N.P.N., blood and urine pH, chloride, sodium, potassium, bicarbonate, and calcium, urine magnesium, and the electrolytes of the whole ashed alligator and alligator feces.

We are indebted to Mr. Rafael C. Sanchez and Miss Lucy C. Gremillion for technical aid.

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19. Unpublished observations.

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Appearance of an Antidiuretic Substance in the Urine of Man after Various Procedures. (17628)

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Although there are many reports on the liberation of an antidiuretic substance in the body after dehydration, emotional stress, electrical stimulation and fainting, there are few studies on man of the excretion in the urine of antidiuretic substances. Under most conditions where an antidiuretic effect is noted in the body, the evidence supports the theory that stimulation of the posterior lobe of the pituitary takes place with a resulting release of antidiuretic substance. This preliminary report describes the extraction of an antidiuretic substance from the urine in various conditions and suggests that this substance is due to posterior lobe activity.

Methods. Antidiuretic assay has been conducted by the method of Burn(1) using the 50% urine excretion of the hydrated rat as the standard. Extracts were administered by both subcutaneous and intraperitoneal injection. Some extracts may be toxic and cause an apparent antidiuretic action only on intraperitoneal injection. The results from extracts which show such an effect are therefore discarded and are considered non-specific. The zinc ferrocyanide method for adsorbing posterior lobe hormones from urine described in 1939(2) has continued to yield excellent results.

Results. 1. *Recovery of posterior pituitary extract added to urine.* Extracts of urine from normal individuals do not exhibit any antidiuretic activity even though concentrates equivalent to 24 hour samples are prepared. The extraction process is effective in recovering from 85-100% of the activity when from 1 to 10 units of posterior lobe extract is added to 1 litre of normal urine. For quantitative estimations it is necessary to inject the extracts intraperitoneally. Subcutaneous injections show an apparent increased potency due to the effects of augmentation by substances in the urine extract(2).

2. *Excretion of intravenous posterior pituitary extract.* The urine from 9 subjects who had received doses of 2 to 5 units of pitressin intravenously† has been extracted and assayed. In all cases an antidiuretic substance appeared in the urine and was completely excreted within 3 hours. Quantitative studies on 7 subjects showed a recovery of from 4.8 to 23%, average 11.2%, of the injected pitressin.

3. *Dehydration.* Partial dehydration of 3 subjects has been accomplished by reducing all free fluid intake and restricting the diet to solid foods. All urine samples obtained after 16 to 23 hours of dehydration exhibited antidiuretic activity. In 1 case it was estimated that after 33 hours of dehydration the secretion of posterior lobe hormone by the pituitary was 6.5 mU/kg/min and this increased to 8 mU after 48 hours of dehydration.

4. *Venesection and Fainting.* Seven subjects† were subjected to experimental venesection and transfusion. None of these fainted. Urine samples obtained throughout the experiment did not contain antidiuretic activity. Seven other subjects subjected to the same procedure fainted at some time during the experiment. Urine samples obtained after the faint in all cases contained an antidiuretic substance. Three persons (not associated with the above experiments) who fainted spontaneously also exhibited antidiuretic activity in urine samples collected immediately after the faint.

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* This research was supported by a National Research Medical Fellowship, Ottawa.

1. Burn, J. H., *Biological Standardization*, Oxford Press, 1939.

2. Noble, R. L., Kinderknecht, H., and Williams, P. C., *J. Physiol.*, 1939, v96, 293.

† These experiments and those on subjects subjected to hemorrhage were made possible through the cooperation of Dr. O. G. Edholm, Department of Physiology, and his collaborators.

5. *Blackout.* The urines from 9 subjects[‡] who lost consciousness due to experimental acceleration (blackout rating of from 4.0 to 6.5 G) were examined. In no case was antidiuretic activity demonstrated in the urine.

6. *Electro-Convulsive Therapy.* Pooled urine samples were obtained from 3 separate groups of psychotic patients before and after E.C.T. The control specimens were devoid of activity, but following therapy marked antidiuretic activity was found.

7. *Eclampsia.* The urine of 7 cases of pre-eclampsia and of one eclamptic was examined and did not contain antidiuretic activity. The negative findings were not related to the associated albuminuria.

Discussion. The results which have been described show that an antidiuretic substance appears and may be extracted from the urine following certain procedures in man. The evidence, although indirect, indicates that this may be the posterior pituitary antidiuretic hormone. A more detailed discussion of these findings and the possible physiological mechanisms involved will be presented in a later paper(3).

Summary. An antidiuretic substance may be extracted from human urine following the intravenous injection of posterior lobe hormone, dehydration, fainting and electro-convulsive therapy.

The urine of normal persons, and subjects following loss of consciousness associated with "blackout" does not contain antidiuretic activity under the experimental conditions described. The urine of eclamptic women was also inactive.

[‡] These experiments were conducted through collaboration with Dr. W. R. Franks, R.C.A.F. Institute of Aviation Medicine, Toronto.

3. Noble, R. L., Plunkett, E. R., and Taylor, N. B. G., *Rec. Advances Hormone Res.*, Vol. IV, Academic Press, N.Y., 1949.

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Production of a Hemorrhagic State by the Infusion of Hemolyzed Blood.* (17629)

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While conducting experiments relative to the development of a mechanical pump-oxygenator for the temporary deviation of blood from the heart and lungs of dogs, hemolysis was frequently encountered. At this time, dogs died of a previously undescribed hemorrhagic state which closely simulated a condition seen on occasion in man. It was therefore deemed advisable to devise a more simple method of reproducing this condition. Since that time, changes in pump-oxygenator have been effective in eliminating hemolysis, and the incidence of this syndrome has been diminished.

This entity is characterized by prolonged or infinite blood coagulation time, and poor clot retraction. This blood will clot *in vitro* by the addition of small amounts of thromboplastin or fresh blood from another dog. This reversal is only temporary *in vivo*, however. Hemorrhage occurs from the entire gut which is contracted, pitted, granular and is studded throughout with petechiae. Bloody effusions invade the serous cavities. Petechiae also appear in the lungs, serosal surfaces and along blood vessels. Microscopically the liver and spleen show parenchymal congestion with erythrocytes and pigment and the kidney tubules and glomeruli also contain pigment. Portions of this syndrome

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[†] Senior Public Health Service Research Fellow of the National Heart Institute.

TABLE I.
Infusion of Heparinized Whole Blood, 3 Hours to 3 Days Old.

					Coagulation time			
Dog	Vol. blood exchanged cc/kg	Method of hemolysis	Amt hemoglobin infused, g/kg body wt	Syndrome	Max.	Hr after infusion	Subsequent min.	Hr after infusion
1	70	Hemolyzing pump	4.1	+	∞	¼	∞	[*]
2	105	,, ,,	3.3	+	22′	1	19′	½
3	118	Shaking	2.2	+	∞	¾	∞	
4	123	*Not* hemolyzed	0.2	—	16′	½	5′	2

[*] Coagulation time temporarily reduced to three min. by thromboplastin intravenously.

TABLE II.
Infusion of Citrated Blood, Twenty-six to Thirty-four Days Old.

			Components present				
Dog	Vol. infused cc/kg	Method of hemolysis	Hemoglobin	Stroma	Amt plasma Hbg. infused g/kg	Syndrome	Max. coagulation time
5	32	Shaking	+	+	1.44	+	2½ hrs
6	36	Distilled water	+	0	2.9	+[*]	2½ hrs
7	44	*Not* hemolyzed			0.012	—	4 min.
8	21	Stroma in saline (no blood)	0	+	0.0	—[†]	4 min.

[*] No serosal or perivascular petechiae.
[†] Petechiae but no hemorrhage or increase in coagulation time.

have been observed as Barratt and Yorke(1) found that larger doses of hemolyzed blood were tolerated and sudden death avoided if stroma was removed from hemolyzed blood. They also noted aberrations in clotting time. Flink(2) observed in one dog the same gross pathology that we report but made no observations on coagulation time.

Methods. All dogs were anesthetized with pentobarbital sodium or pentothal and cross matched. Donor blood was drawn from normal animals using 30 mg heparin or 240 cc A.C.D.[‡] solution per liter for anticoagulant as indicated. Hemolysis was produced in one of 3 ways: (1) blood was run for 20 minutes through a pump known to produce hemolysis, (2) blood was shaken manually until plasma hemoglobin reached desired level, (3) refrigerated citrated blood was treated by decanting the plasma and washing the red blood cells 3 times with isotonic saline. One hundred cc distilled water were added to 200 cc red blood cells with centrifugation for one hour at approximately 2000 r.p.m. to separate the stroma from the supernatant fluid. In Experiment I, arterial blood was withdrawn in volume equal to that of the intravenous infusion. Coagulation times were determined by a modified Lee-White method; plasma hemoglobins were done by the acid hematin and oxyhemoglobin methods.

Results are shown in Tables I and II.

Discussion. Hemolysis does not appear to be the only factor in operation, as this syndrome has been observed in dogs with a lower plasma hemoglobin than that in certain other surviving animals. Plasma hemoglobin alone is apparently not the sole cause as I.V. administration of distilled water sufficient to raise plasma hemoglobin above 1.0 gram per cent will not cause this condition. Studies to elucidate etiological factors are being pursued in our laboratory at the present time.

Summary. A syndrome of prolongation of blood coagulation time, gastro-intestinal bleeding and petechiae is described. This condition can be produced in the dog by the infusion of hemolyzed blood.

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1. Barratt, J. O. W., and Yorke, W., *Brit. M. J.*, 1914, v1, 235.

2. Flink, E. B., *J. Lab. and Clin. Med.*, 1947, v32, 223.

[‡] A.C.D. solution contains 2.45% dextrose, 1.37% sodium citrate, 0.50% citric acid in distilled water.

Effect of Toxic Doses of 4,4′-Diamidino-Stilbene (Stilbamidine) on the Electrocardiogram in Mice. (17630)

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Diamidine compounds upon rapid intravenous administration produce severe cardiovascular symptoms. Studies on simultaneous multiple electrocardiographic and pulse tracing in patients with multiple myeloma injected with 4,4′-diamidino-stilbene (stilbamidine)* revealed changes indicative of possible myocardial or peripheral origin associated with tachycardia and hypotension(1). To investigate the cardiac effects of the drug in the toxic range, stilbamidine was given parenterally to mice and the effects were observed in three simultaneous electrocardiograms.

Method. Twelve experiments were conducted using Strain A mice, 5 to 10 months old, and approximately 25 g in weight. Standard leads I, II and III were taken, employing needle electrodes inserted subcutaneously into the appropriate extremities of restrained but unanesthetized mice. The impulses were amplified and applied to mirror-type galvanometers† in a recording oscillograph. The tracings were made simultaneously, with the recording paper moving at 8 inches per second.

The reproducibility of electrocardiograms was determined in 16 mice by taking frequent records over a period of 6 weeks. After at least 2 control records had been taken, stilbamidine dissolved in sterile distilled water was injected intraperitoneally. Records were taken continuously during the first 5 minutes after injection and at progressively longer intervals thereafter. The materials were injected intraperitoneally in all studies.

Results. Intraperitoneal injections of 2 mg (80 mg/kg) or less of stilbamidine did not

TABLE I.
Effect of Intraperitoneal Stilbamidine in Mice.

Mouse No.	Stilbamidine		Effect	
	mg	mg/kg	On electrocardiogram	Mortality
1	1.2	48	No significant change.	Survived.
2	2.5	100	Slight transient sino-auricular depression.	”
3	2.5	100	Shift in auricular pacemaker.	”
4	4	160	Progressive reverse Wenkebach; shifting pacemaker; A-V nodal rhythm. Rate slowed to 350/min. in 20 min.	Died overnight.
5	4	160	Complete A-V dissociation with I-V conduction defect.	Respirations ceased, 5.5 min.
6	5	200	Shifting pacemaker, progressive A-V block, to final bundle branch block and complete A-V dissociation.	” ” 4 ”
7	5	200	Progressive A-V, S-A, and I-V block to complete A-V dissociation.	” ” 4.5 ”
8	5	200	Flattening of T wave with slowing of rate to 480/min. within 30 min.	Died within 24 hrs.
9	5	200	Flat T waves, rate slowed to 600/min. in 25 min.	” ” ” ”
10	5	200	Abrupt slowing of rate at 10.5 min.; 6:1 A-V block at 16 min.	Died, 35 min.
11	10	400	Progressive S-A, A-V, and I-V block.	Respirations ceased, 4 min.
12	20	800	” ” ” ” ” ”	” ” 3.5 ”

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* Furnished by Merck and Co., Inc., Rahway, N.J.

1. Bierman, H. R., and Sokolow, M., *J. Nat. Cancer Inst.*, 1949, v10, 279.

† No. 7-120-CEC, Pasadena, Calif. Frequency response 0-500 cps.

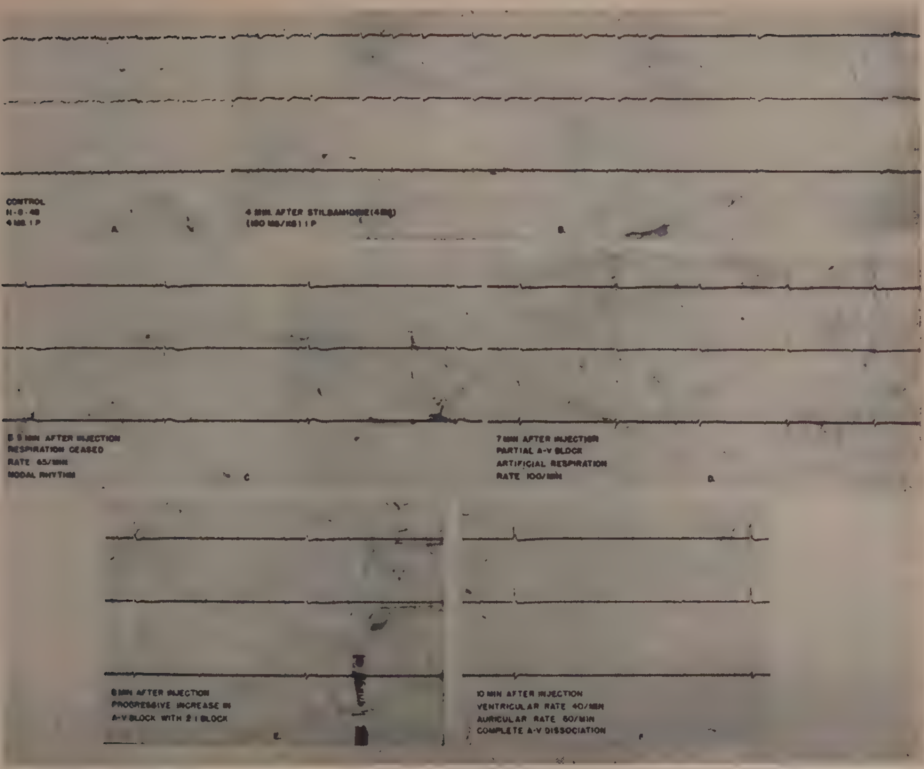

FIG. 1.

Standard leads I, II, and III recorded simultaneously on Strain A mouse following 4 mg intraperitoneally of stilbamidine (160 mg/kg of mouse). Note progressive slowing of rate and development of A-V block. Ventricular complex contour is maintained.

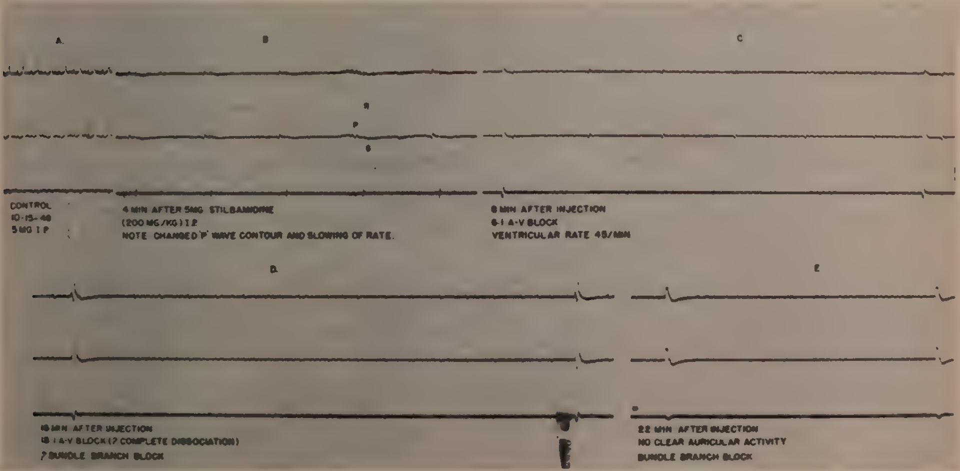

FIG. 2.

Simultaneous electrocardiographic leads I, II, and III on Strain A mouse. Note progressive development of A-V block and change in auricular and ventricular complex contour.

produce any toxic signs or electrocardiographic changes. Transient and minor electrocardiographic changes were observed with doses of 2.5 mg (100 mg/kg). Doses above 5 mg (200 mg/kg) were lethal. Striking electrocardiographic abnormalities followed by cessation of respiratory and cardiac activity occurred with 4 to 20 mg (160 to 800 mg/kg) in most animals (Table I). Respirations usually stopped within 4 to 6.5 minutes, and cardiac action usually ceased approximately 18 minutes after intraperitoneal injection with higher doses. Artificial respiration was not effective when respirations had ceased.

The most striking electrocardiographic findings following the intraperitoneal administration of stilbamidine were progressive and varying degrees of sino-auricular, auriculo-ventricular, and intraventricular block, occurring in that order. During the development of sino-auricular block, the cardiac rates slowed from the usual control rate of 600 per minute to as low as 10 per minute (Fig. 1).

Varying degrees of auriculo-ventricular block from 10 to 1 to complete auriculo-ventricular dissociation were noted (Fig. 2). After respirations had ceased and the cardiac rate had slowed materially, intraventricular and possibly bundle-branch block occurred. There was no discordant relationship noted between any of the 3 electrocardiographic leads following lethal doses of stilbamidine. The electrocardiographic changes preceded respiratory cessation by 2 to more than 30 minutes. Anoxia following the respiratory cessation may contribute to the late electrocardiographic changes.

Conclusions. 1. Stilbamidine administered intraperitoneally to mice in doses of 4 to 20 mg (160 to 800 mg/kg) caused cessation of respirations in 4 to 6.5 minutes, accompanied by progressive and varying degrees of sino-auricular, auriculo-ventricular and intraventricular block eventuating in death. No significant ST-T changes occurred.

2. The constancy and reproducibility of the electrocardiogram in the normal mouse indicates that the effect of drugs upon the electrocardiogram may profitably be studied in this animal.

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Relationships of Availability of Oxygen to Physical Fitness in Patients with Cardio-respiratory Diseases.* (17631)

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The concept of physical fitness in either normal subjects or patients with various cardio-respiratory diseases refers to the integrated capacity of the heart, lungs, blood and regulatory mechanisms to meet exertional demands. Fitness is measured by the least variable characteristics of the cardio-respiratory responses to exercise(1). The latter is standardized by walking on a motor-driven treadmill at a fixed velocity; hence the rate of energy expenditure is proportional to the body weight(2). Although this work load is only moderate for normal subjects, it may be

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[†] Bertha Hochstetter Buswell Research Fellow in Medicine.

1. Bruce, R. A., Pearson, R., Lovejoy, F. W., Jr., Yu, P. N. G., and Brothers, G. B., *J. Clin. Invest.*, 1949, v28, 1431.

2. Bruce, R. A., Lovejoy, F. W., Jr., Pearson, R., Yu, P. N. G., Brothers, G. B., and Velasquez, T., *J. Clin. Invest.*, 1949, v28, 1423.

exhaustive for patients with functional impairment due to disease. The physical fitness index equals:

$$\frac{\text{average respiratory efficiency during exercise} \times \text{duration of exercise[‡]} \times 100}{\text{total heart beats for first 3 minutes of recovery (3)}}$$

Normal values range between 12 and 26, with a mean value of 19 in subjects walking for 10 minutes at 2.60 m.p.h. Consecutive chest lead electrocardiograms show abnormalities of the ST-T waves, corrected QT intervals, and QT/TQ ratios only in patients with disease(4).

Various resting measurements of cardio-respiratory function, such as estimations of the lung volumes, cardiac output, etc. contribute towards the understanding of the pathological physiology in patients with disease. The aim of this report is to compare and assess the relative value of several of these laboratory methods to the performance of activity, as measured by the physical fitness index. For these purposes, an heterogeneous group of patients has been assembled. They varied in age between 7 and 70 years, and in disability from virtually none to total.

Methods. Twenty-nine patients on whom multiple laboratory measurements have been made, and 3 who were studied again under clinically different circumstances resulting from therapy were included in this study. The chief clinical categories of disease represented were: congenital cardiovascular disease 12, acquired heart disease 7, chronic pulmonary disease 9, and miscellaneous diseases affecting cardio-respiratory function (mediastinal tumor, constrictive pericarditis) 4. Resting measurements were made of respiratory gas exchange, blood gas composition, lung volumes (by helium dilution technique(5)), and cardiac output by the direct Fick principle. Treadmill exercise tolerance was evaluated at (level) walking rates of 2.6 m.p.h., and at 1.73 m.p.h. for those patients who were unable to walk at the former pace[§]. Statistical correlations were made by the product-moment method(6), and scatter graphs of significant relationships are included. The effective alveolar ventilation has been derived from the ratio of

$$\frac{\text{Insp. } O_2\% - \text{Exp. } O_2\%}{\text{Insp. } O_2\% - \text{Ideal Alv. } O_2\%}$$

times the observed minute ventilation volume. The effective alveolar perfusion has been derived from the cardiac output and the difference between the pulmonary venous and the mixed venous oxygen content, assuming at least 95% saturation of the former blood value. The "ideal" alveolar pO_2 has been derived from the arterial pCO_2 and expired air R.Q.(7).

Results. Table I presents the statistical relationships between the physical fitness index of exercise tolerance and various respiratory measurements in 29 patients with disease. Figure 1 shows that the physical fitness score tends to vary inversely with the observed mid-capacity pO_2-mixed arterial gradient and directly with the mean capillary pO_2 of the tissues of the body[‖]. Despite large co-

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[‡] To the limits of tolerance, or an arbitrary maximum of 10 minutes.

3. Bruce, R. A., Lovejoy, F. W., Jr., Yu, P. N. G., Pearson, R., and McDowell, M., *Science,* 1949, v110, 442.

4. Yu, P. N. G., Bruce, R. A., Lovejoy, F. W., Jr., and Pearson, R., *J. Clin. Invest.,* 1950, in press.

5. Meneely, G., and Kaltreider, N. L., *J. Clin. Invest.,* 1949, v28, 129.

[§] More satisfactory correlations would be demonstrated if all the patients were able to walk at the rate of 2.60 m.p.h., even for a short period of time. Patients with *intermediate* functional capacity may be able to endure exercise for 10 minutes at 1.73 m.p.h., but not able to continue for as long as that at 2.60 m.p.h. The lower fitness score, due to the shortened time, would be significantly different from that obtained for the slower rate. Since 9 of the 12 patients included here did walk 10 minutes at 1.73 m.p.h., some of them might fall within the above category. Unfortunately comparative studies on these particular patients were not made.

6. Pearson, F. A., and Bennett, K. R., *Statistical Methods,* John Wiley and Sons, Inc., 1942.

7. Riley, R. L., and Cournand, A., *J. Appl. Physiol.,* 1949, v1, 825.

[‖] The mid-capacity pO_2 is the terminal pO_2 of expired air (at the mid-capacity level of pulmonary capacity. It differs from the Haldane-Priestley alveolar pO_2 in that the reserve volume

TABLE I.
Relationships of Physical Fitness Index of Exercise Tolerance and Various Resting Physiological Factors in Cardio-Respiratory Diseases.

Factor		Mean ± S.D.	Range	Coefficient of variation, %	Coefficient of correlation*
Observed Mid-capacity pO_2-mixed arterial pO_2 gradient	mm of Hg	43.7 ± 21.1	9-98	48.2	—.634 ± .10
MC pO_2-"Ideal" alveolar pO_2	"	21.3 ± 8.1	2-35	38.0	—.417 ± .14
"Ideal" alveolar pO_2-m. art. pO_2	"	26.8 ± 20.6	(—11)-57	76.9	—.421 ± .14
Inspired pO_2-"Ideal" alveolar pO_2	"	50.5 ± 8.5	33-67	16.8	—.109 ± .11
Mixed Arterial pO_2	"	71.8 ± 17.1	37-102	23.8	+.641 ± .10
Mean Capillary pO_2	"	46.5 ± 9.6	26-61	20.6	+.675 ± .09
Mixed Venous pO_2	"	34.6 ± 6.6	18-49	19.1	+.542 ± .12
A — V pO_2 difference	"	37.0 ± 15.3	9-83	41.4	+.540 ± .13
A — V O_2 content diff.	ml/l	50.2 ± 18.0	14-94	35.8	—.013 ± .18
% Mid-capacity Vol.		50.3 ± 13.5	32-83	26.8	—.262 ± .17
% Residual Air Vol.		35.9 ± 10.4	20-71	29.0	+.101 ± .18
% Effective Alveolar Ventilation		47.8 ± 12.2	30-89	25.5	+.325 ± .15
% Effective Alveolar Perfusion		81.5 ± 14.4	49-97	17.7	+.353 ± .15
Cardiac Index	l/M²/min.	3.29 ± 1.09	1.5-6.4	33.1	+.086 ± .17
Oxygen Index		4.03 ± 2.1	1.5-9.8	52.1	+.087 ± .17

* Standard Error of Coefficient of Correlation equals $\frac{1 - r^2}{\sqrt{N - 1}}$

efficients of variation in the several measurements obtained from this heterogeneous group of patients, moderately significant correlations were obtained for each of these relationships. Less significant, or even doubtful correlations, were obtained for the relations of fitness for exercise to the subdivisions of the observed oxygen gradient. For example the "ideal" alveolar pO_2-mixed arterial pO_2 gradient showed a poor correlation ($r = -0.421 \pm .143$). Somewhat more significant correlations were obtained between the fitness score of exercise tolerance and various pO_2 values, especially the arterial blood, but also mixed venous blood, and even the arterio-venous pO_2 difference in mm of mercury. In contrast to the last finding, there was no relationship between fitness and the arterio-venous oxygen difference expressed as contents in volumes percent.

Although the percentage residual air volume, or functional residual air volume (mid-capacity volume), effective alveolar ventilation, effective alveolar perfusion, cardiac index (cardiac output per square meter of body surface area), or oxygen index of supply to demand (arterial oxygen content divided by A – V oxygen difference(9)), commonly deviate from the normal values in patients with cardio-respiratory diseases, none of these *resting* measurements correlated significantly with exercise tolerance¶ (Table I).

It was noteworthy that both the observed mid-capacity pO_2 to mixed arterial pO_2 gradient, and the mean tissue capillary pO_2,

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is not forcibly expired, and it represents the terminal mixture of air from both functioning and non-functioning alveoli (alveoli ventilated but not adequately perfused). The mixed arterial pO_2 is that obtained from systemic arterial blood. It is the mixture of pulmonary venous blood and variable amounts of venous admixture of unoxygenated blood (including alveoli perfused but not adequately ventilated). The mean capillary pO_2 is derived from Barcroft's formula(8), and equals the mixed venous $pO_2 + \frac{A - V\ pO_2 \text{ difference}}{3}$. The mixed venous pO_2 has been estimated from the observed oxygen saturation and carbon dioxide content values of the blood from the right heart, and the oxygen dissociation curve for blood.

8. Barcroft, J., *Factors in the Architecture of Physiological Function,* Macmillan Co., Cambridge, England, 1934, 219.

9. Little, J. M., *Am. J. Med.,* 1949, v7, 207.

¶ This need not, of course, apply to *homogeneous* groups of patients, such as predominantly emphysema, or congestive heart failure.

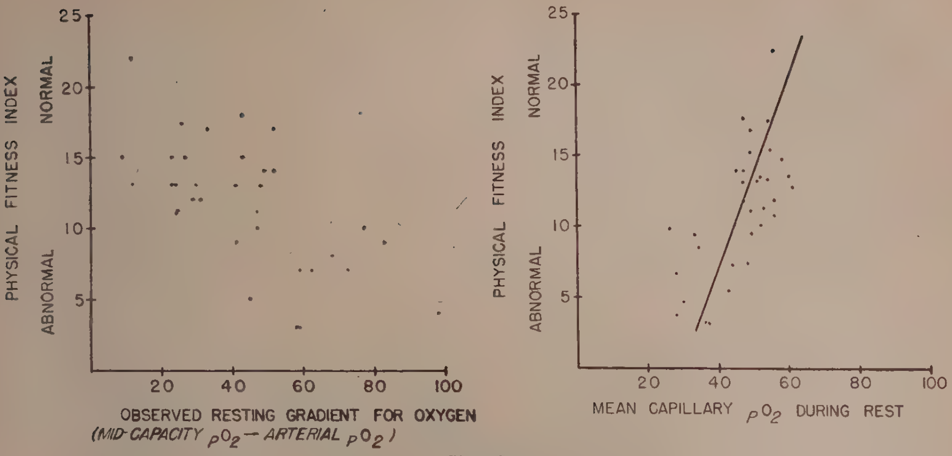

FIG. 1.

Relationship of physical fitness indices of patients with various cardio-respiratory diseases to the observed mid-capacity pO_2 to mixed arterial pO_2 resting gradient, and the calculated mean tissue capillary pO_2 during rest.

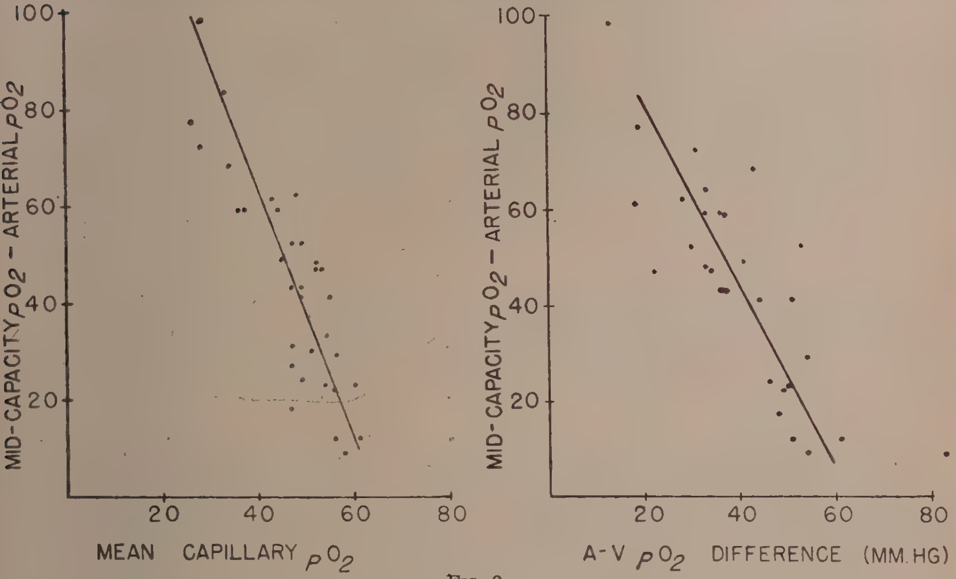

FIG. 2.

Relationship of the observed resting mid-capacity pO_2 to mixed arterial pO_2 gradient (of the same patients) to the calculated mean tissue capillary pO_2, and the arterio-venous pO_2 difference during rest.

which exhibited moderately significant relationships to fitness for exercise, were definitely related to each other (Fig. 2). As the observed oxygen gradient widened, the capillary pO_2 diminished ($r = -0.881 \pm .039$). Furthermore there was a significant relationship between the observed oxygen gradient and the A – V pO_2 difference

$$(r = -0.801 \pm .067).$$

The latter relationship is largely dependent upon the oxygen dissociation curve.

Comments. Assuming that the physical fitness index is generally a satisfactory measure of clinical exercise tolerance, one then has a quantitative measure which may be compared with other laboratory data. Such comparisons have been made on an heterogeneous group of patients in order to test the relationships under unfavorable circumstances. If physiological relationships can be demonstrated, they should be applicable to various types of cardio-respiratory pathology. Three such relationships, of moderate significance, have been demonstrated by these analyses. One is that exercise tolerance varies inversely with the magnitude of the resting observed gradient of the lungs (mid-capacity pO_2 - mixed arterial pO_2). The others are that exercise tolerance varies directly with the estimated mean tissue capillary pO_2 derived from Barcroft's formula and the arterial pO_2. Furthermore, the observed gradient and the mean capillary pO_2 are closely related to each other in an inverse manner. The observed gradient also varies inversely with the $A - V$ pO_2 difference (in relation to the oxygen dissociation curve for blood). Hence exercise tolerance is dependent upon the availability of oxygen to the tissues. Possibly correlations of higher significance would be derived if suitable measurements were made serially during the performance of exercise. Although the recognition of hypoxemia during rest suggests reduced tolerance for exertion, the best measure of this loss of reserve is the determination of exercise tolerance itself, because there is no assurance that the hypoxemia evident at rest will not be increased by exertion. Inasmuch as oxygen is neither absorbed nor distributed unless the heart is circulating the blood, the ultimate limiting factors of exercise tolerance are circulatory in nature. Finally many of the factors which may influence the magnitude of the observed oxygen gradient in patients with cardio-respiratory diseases may fail to exhibit, from resting measurements, adequate predictions of exercise tolerance, or working capacity.

Summary. Significant correlations were obtained in patients with diverse cardio-respiratory diseases, and varying disability, between the oxygen gradient of the lungs, the oxygen tension of the peripheral tissues during rest, and the physical fitness index of exercise tolerance. Working capacity is proportional to the availability of oxygen to the tissues, and the limiting factor is circulatory in nature.

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Aspartic Acid Decarboxylase in Bacteria.* (17632)

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Billen and Lichstein(1) presented evidence for the existence of an aspartic acid decarboxylase in *Rhizobium trifolii.* The present report is an extension of these studies with *Rh. trifolii* and other species of microorganisms.

Methods. The organisms were grown in a medium of the following composition: 1% each of yeast extract and tryptone, 0.6% glucose, 0.5% dipotassium phosphate, and 0.1% *DL*-aspartic acid. Incubation was carried out for 16-18 hours at 30°-37°C depending on the optimum for the organism. Cells were harvested by centrifugation, washed twice with distilled water, and concentration determined in terms of bacterial nitrogen per ml of suspension by measuring turbidity in a Klett-Summerson photoelectric colorimeter and converting into terms of nitrogen content by use of previously standardized graphs. Dried cells were prepared by drying the washed cell suspensions *in vacuo* over Drier-

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* Aided in part by RG 1581 of the Division of Research Grants and Fellowships of the National Institutes of Health, United States Public Health Service.

1. Billen, D., and Lichstein, H. C., *J. Bact.*, 1949, v58, 215.

TABLE I.
Distribution of Aspartic Acid Decarboxylase in Bacteria.*

Organism	Mg cell nitrogen	*beta*-alanine produced (µg)	$Q_{(N)}$-*beta*-alanine†	Pantothenic‡ acid requirement
E. coli (Tenn.)	.55	2.4	4.3	—
” (Tex.)	.84	7.3	8.6	—
” (Gratia)	1.31	3.6	2.7	—
P. vulgaris	1.43	1.6	1.1	—
Bact. cadaveris (Gale)	1.56	1	.6	—
Az. vinelandi	.97	6	6.2	—
Rh. trifolii	1.72	11.7	6.8	—
Sachh. cerevisiae	.45	0		+
” *fragilis*	.89	0		+
” *ellipsoideus*	.43	0		+
C. diphtheriae	.47	0		+
S. aureus	1.22	0		—
A. aerogenes	1.13	0		—
L. casei	1.26	0		+
L. arabinosus	.66	0		+
S. faecalis	1.36	0		+

* 60 min., 37°C, pH, 5.5, *L*-aspartic acid substrate.

† $Q_{(N)}\text{-}beta\text{-alanine} = \dfrac{\mu g\ beta\text{-alanine}}{\text{time} \times \text{mg nitrogen}}$

‡ + indicates failure to grow in chemically defined media without added pantothenic acid.
— indicates ability to grow in chemically defined media without added pantothenic acid.

ite. Cell-free juices were prepared by autolysis at pH 8, M/10 phosphate, at 37°C for 24 hours followed by high speed centrifugation to yield a clear supernate.

At the conclusion of an experiment the tubes were immersed in boiling water for 5 minutes to stop the reaction, the tubes centrifuged, and an aliquot of the supernate assayed for *beta*-alanine(1) employing *Saccharomyces fragilis* (ATC No. 2360). For the microbiological assay of *alpha*-alanine the method of Sauberlich and Baumann(2) using *Leuconostoc citrovorum* (ATC No. 8081) was employed.

Results. Inasmuch as Billen and Lichstein(1) had reported positive activity only with *Rh. trifolii* and *Escherichia coli* (Texas), it seemed desirable to screen a number of different organisms for their ability to decarboxylate aspartic acid. It may be seen (Table I) that of 16 strains tested only 7 exhibited positive activity. The limited distribution of this decarboxylase agrees well with that of other amino acid decarboxylases(3). It was thought possible that the limited activity found in all the positive strains could be due partially to concurrent decarboxylation to yield *alpha*-alanine. This was investigated in *Rh. trifolii*, but in no experiment was any activity demonstrated for an aspartic acid decarboxylase to yield *alpha*-alanine rather than *beta*-alanine.

The enzyme is quite stable to vacuum drying yielding preparations having an activity comparable to that found in living cells. At pH 6, 90 minutes incubation, 10 mg dried cells of *Rh. trifolii* per tube, the following values for *beta*-alanine production at various temperatures were recorded: 20° = 0.8 µg; 30° = 4.0 µg; 40° = 7.6 µg; 45° = 6.0 µg. Thus, the optimum temperature appears to be 40°C in contrast to 46°C for the living cell. Employing the same preparation at 38°C the following values for different pH's were noted: pH 3 = 2.0 µg; pH 4 = 5.6 µg; pH 5 = 7.0 µg; pH 6 = 8.4 µg; pH 7 = 3.2 µg; pH 8 = 0 µg. These data suggest an optimal pH of 6 as in the living cell. Although no extensive work was done, the enzyme is reasonably stable to autolysis which yields a cell-free juice from *Rh. trifolii* having a Q value of 2-3 in contrast to 5-7 for the living cell.

Discussion. The results of these experi-

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2. Sauberlich, H. E., and Baumann, C. A., *J. Biol. Chem.*, 1949, v177, 545.
3. Gale, E. F., *Adv. in Enzymology*, 1946, v6, 1.

ments provide evidence for the presence of an enzymatic aspartic acid decarboxylase, yielding *beta*-alanine, in several species of microorganisms. The data also show that this enzyme is not widely distributed among bacterial species, and when present exhibits a relatively low activity. If aspartic acid decarboxylase is concerned only with the maintenance of an adequate amount of *beta*-alanine for the synthesis of pantothenic acid, the low activity is in keeping with the small amount of this vitamin usually necessary for optimal growth. Further, the enzyme may be present only in those organisms which synthesize *beta*-alanine, and conversely failure to synthesize *beta*-alanine or pantothenic acid may be an indication of the absence of aspartic acid decarboxylase. In order to test this hypothesis the pantothenic acid requirements of the organisms employed here were studied by employing chemically defined media. It may be seen (Table I) that in keeping with this hypothesis all of the organisms exhibiting positive aspartic acid decarboxylase activity are capable of growing in synthetic media without added *beta*-alanine or pantothenic acid suggesting that they are able to synthesize their own supply. On the other hand, 2 of the 9 strains exhibiting negative activity with respect to aspartic acid decarboxylase, namely *A. aerogenes* and *S. aureus,* are capable of growing in a medium devoid of pantothenic acid. It appears therefore that the correlation between ability to synthesize pantothenic acid and the presence of an aspartic acid decarboxylase is not without exception.

Summary. Of 16 strains of microorganisms studied, 7 exhibited positive aspartic acid decarboxylase activity. Employing *Rh. trifolii* this enzyme was found to be stable to vacuum drying, and active cell-free juices could be prepared.

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Effectiveness of Various Drugs in Prevention of Airsickness. (17633)

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The effectiveness of β-dimethylaminoethyl benzohydryl ether 8-chlorotheophyllinate (Dramamine) in preventing sea(1,2) and airsickness(3) has focused attention on other drugs with similar chemical or pharmacological properties. Since Dramamine is the 8-chlorotheophylline salt of β-dimethylaminoethyl benzohydryl ether (Benadryl) the question immediately arises whether the effectiveness resides in either component or only in the combined form. We have investigated this problem in the airplane, using the simulated turbulence technic of Strickland and Hahn(3). Benadryl and 8-chlorotheophylline were each given in 50 mg doses one hour before take-off.

Table I shows that Benadryl possesses marked protective action against airsickness. There was no significant difference between the protection afforded with Benadryl and

TABLE I.
Effect of Preflight Medication on Airsickness.

Preflight medication	Subjects	Airsick (vomited)	
		No.	%
Placebo	75	40	53.3
Benadryl	75	23	30.7
Placebo	78	52	66.7
8-Chlorotheophylline	78	39	50.0
Placebo*	108	60	55.6
Dramamine* (100 mg)	108	31	28.7

* Data from Strickland and Hahn(3).

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1. Gay, L. N., and Carliner, P. E., *Bull. Johns Hopkins Hosp.*, 1949, v84, 470.
2. Gay, L. N., and Carliner, P. E., *Science,* 1949, v109, 359.
3. Strickland, B. A., and Hahn, G. L., *Science,* 1949, v109, 359.

TABLE II.
Comparison of Hyoscine-Benadryl Mixture with Hyoscine Alone.

Preflight medication	Subjects	Airsick (vomited) No.	%
Hyoscine	135	26	19.3
Hyoscine-Benadryl	135	14	10.3

that with Dramamine. Slight protection was given by 8-chlorotheophylline. It is interesting to note that in those instances where Benadryl was unable to prevent vomiting the time of onset was nevertheless delayed over that of the placebo group (34.2 to 29.0 minutes).

Hyoscine hydrobromide has long been the drug of choice in airsickness(4). Because of the difference in pharmacological action of hyoscine and Benadryl, it was thought that a combination of these compounds might be more effective than either alone. Therefore, one hour before flight hyoscine hydrobromide alone was given to one group of subjects in 0.65 mg doses, while a mixture of 0.65 mg hyoscine hydrobromide and 50 mg Benadryl was given to a second group.

Table II indicates the superiority of the mixture. Only 10.3% of the subjects in this group became sick, compared with 19.3% with hyoscine alone, approximately 30% with Benadryl or Dramamine and over 50% with placebos. In every instance significance has been determined between the control and experimental groups by means of a sequential test of hypothesis. The 8-chlorotheophylline series was truncated after 13 flights so that the level of significance is probably between 7 and 10%. In all other cases truncation was not necessary and the difference between the groups was significant at the 5% level. The incidence of vasomotor disturbances and nausea not leading to vomiting was similarly decreased among those subjects receiving hyoscine-Benadryl. The average time of onset of vomiting was delayed from 29.0 minutes with hyoscine alone to 42.0 minutes with the hyoscine-Benadryl combination. Drowsiness and dryness of the mouth were the chief complaints of those receiving the mixture.

Experiments are under way to test smaller doses and other antihistaminics purported to have fewer side effects.

Summary. No significant difference was found between Benadryl (50 mg) and Dramamine (100 mg) in protecting subjects from airsickness during a turbulent flight in an airplane. 8-Chlorotheophylline (50 mg) gave only slight protection as compared with placebos. A combination of hyoscine hydrobromide (0.65 mg) and Benadryl (50 mg) was found to be more effective than either of these drugs alone.

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4. Smith, P. K., USAF Sch. Aviat. Med. Res. Proj. 468, Rep. No. 1, June 14, 1946.

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Occurrence and Distribution of an Inhibitor for Desoxyribonuclease in Animal Tissues.* (17634)

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The presence of a specific inhibitor for desoxyribonuclease in the crop gland of brooding pigeons has recently been described(1). The purpose of the present work was to investigate the distribution of the inhibitor in

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† Some of the data included in this report were taken from a thesis submitted by Mr. E. J. Cooper to the Graduate School of Marquette University in partial fulfillment of the degree of Master of Science. Present address: Carnation Company Research Laboratories, Milwaukee.

several animal tissues, bearing in mind that it was first found in rapidly growing epithelium.

Experimental. Desoxyribonucleic acid was prepared from calf thymus according to Hammersten(2). Once recrystallized desoxyribonuclease (DRNase) was prepared according to Kunitz(3). All experiments were made on the same batch of DRNase. The viscosimetric determination of activity was performed according to Laskowski and Saidel(4), with the exception that variable amounts of inhibitor were added to the reaction system. Unless otherwise specified, the fresh tissue to be examined was ground in the Waring blendor with 10 volumes of water and centrifuged in the cold at 4,000 r.p.m. for 20 minutes.

Early in the course of this work it became apparent that the relationship between the amount of inhibitor and the observed inhibition was not linear. In order to secure even a roughly quantitative method for the determination of the inhibitor, it was necessary to construct an empirical curve. Several attempts in this direction were made, first using the crude extract of veal liver, later a somewhat purified inhibitor from bull testes. Since the same type of phenomenon was observed in both cases, only the results of the latter experiment are presented in Fig. 1. It was arbitrarily decided to call one unit of the inhibitor the amount present in 1 ml of the solution which was used in the experiment shown in Fig. 1. The amount of the inhibitor in an unknown solution was read from the curve, using only its initial part (between 0 and 0.5 ml). The shape of the approximated curve resembled the one found by Kunitz(5) for the inhibition of chymotrypsin α by soybean trypsin inhibitor. It was strikingly different from the direct proportionality found for the system: trypsin, soybean trypsin inhibitor(5). This suggests that the re-

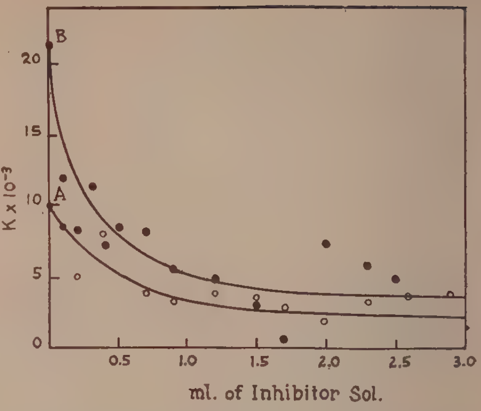

FIG. 1.

The effect of increasing amounts of inhibitor on the activity of desoxyribonuclease. Ordinate: activity of desoxyribonuclease expressed as K (from $K = 1/t \log \frac{\eta_o}{\eta_t}$), abscissa: ml of inhibitor solution added to the viscosimeter. The solution of inhibitor contained 5.0 mg protein per ml. It was obtained by extracting bull testes with 3 volumes of water, fractionating between 30-60% saturation of ammonium sulfate, and finally, dialyzing. Curve B, 1 γ, and Curve A, 0.5 γ, of desoxyribonuclease.

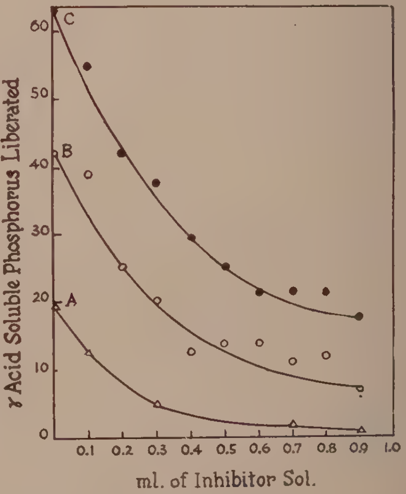

FIG. 2.

The effect of increasing amounts of inhibitor on the activity of desoxyribonuclease. Ordinate: gamma of acid-soluble phosphorus liberated, abscissa: ml of inhibitor solution added to the reaction mixture. The solution of inhibitor was the same as used in the experiments summarized in Fig. 1. Curve A, 0.5 γ; Curve B, 1.0 γ; Curve C, 1.5 γ of desoxyribonuclease.

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1. Dabrowska, W., Cooper, E. J., and Laskowski, M., *J. Biol. Chem.*, 1949, v177, 991.
2. Hammarsten, E., *Biochem. Z.*, 1924, v144, 383.
3. Kunitz, M., *Science*, 1948, v108, 19.
4. Laskowski, M., and Saidel, M. K., *Arch. Biochem.*, 1945, v7, 465.
5. Kunitz, M., *J. Gen. Physiol.*, 1947, v30, 291.

TABLE I.
Distribution of Inhibitor in Pathological Human Tissues.

Case No.	Tissue	Viscosity units/g of fresh tissue
1	Breast adenocarcinoma	25.0
2	” ”	8.3
3	Aberrant breast tissue (4 mo. pregnant)	0.8
4	Bowel adenocarcinoma	16.6
5	Gastric ”	14.0
	Gastric mucosa (normal)	6.7
6	Sternal bone marrow (pernicious anemia)	11.7
	Sternal bone marrow (after Vit. B_{12})	10.0
7	Breast adenocarcinoma	2.0
	Lymph node (normal)	2.0
8	Cancer of breast	2.5
	Lymph node metastasis	1.8
9	Carcinoma of lymph nodes	1.5
	Lymph nodes (normal)	1.6

action between DRNase and the inhibitor is of the reversible type and probably obeys the law of mass action. An identical conclusion was reached from the experiment (Fig. 2) in which inhibition was determined by the acid soluble phosphorus method(6).

Table I shows the results of experiments in which human surgical material‡ was analysed for inhibitor. The content of inhibitor in malignant tissue varied widely from one case to another, while no striking differences were found between the malignant and non-malignant tissues of the same patient.

Regenerating rat livers were next investigated. Partial hepatectomy was performed§ according to Higgins and Anderson(7). No substantial difference in activity of DRNase between the regenerating and the control lobes could be demonstrated. This is in agreement with erratic changes reported by Novikoff and Potter(8) for desoxyribonucleic acid.

Finally several tissues of the normal rat were analysed for both DRNase and the inhibitor. The results of one of these experiments are shown in Table II. In several cases the same organ showed the presence of both enzyme and inhibitor simultaneously. This was most evident in the case of liver. Grinding with sand produced an extract rich in the inhibitor and comparatively poor in enzyme. A more efficient disintegration of the tissue in the Waring blendor resulted in an increased extraction of the enzyme and an apparent decrease of the inhibitor.

Table III shows the results of experiments in which normal rat livers were first “diced” by cutting with scissors, treated with 10 volumes of water, left to stand for 20 minutes at 5°, and centrifuged in the cold at 4,000 r.p.m. The supernatant was removed and analysed for both DRNase and the inhibitor. The tissue was then transferred to an ice-cooled Waring blendor and homogenized with an additional amount (10 volumes) of water. The homogenate was again centrifuged and the supernatant analyzed. The results indicate that the inhibitor is much more easily extractable than the enzyme. With the methods now available, it was not possible to determine the relative amounts of enzyme and inhibitor in the two fractions. It is obvious, however, that the inhibitor strongly predominated in the diced preparation, while the enzyme predominated in the blended.

In view of this finding further fractionation

TABLE II.
Distribution of Desoxyribonuclease and Its Inhibitor in Various Organs of the Rat.

Organ	Viscosity units of enzyme	Viscosity units of inhibitor
Liver (blendor)	5.8	.0
Liver (sand)	2.4	2.8
Kidney (blendor)	24.0	.0
Muscle (blendor)	1.3	.0
Lungs (blendor)	2.8	.8
Testes (blendor)	.0	1.0
Brain (sand)	.4	.0
Spleen (sand)	1.0	.4
Heart (sand)	3.3	.2

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6. Laskowski, M., *Arch. Biochem.*, 1946, v11, 41.

‡ We are greatly indebted to Dr. M. M. Kaser, Dr. J. F. Kuzma, and Dr. M. Schuster for the samples of tissue.

§ We wish to express our appreciation to Mr. E. R. Haushalter for performing the operations.

7. Higgins, G. M., and Anderson, R. M., *Arch. Path.*, 1931, v12, 186.

8. Novikoff, A. B., and Potter, V. R., *J. Biol. Chem.*, 1948, v173, 223.

TABLE III.
Effect of Homogenization on Activities of Extracts.

		"Diced"		"Blended"	
		DRNase (Viscosity units)	Inhibitor (Viscosity units)	DRNase (Viscosity units)	Inhibitor (Viscosity units)
Rat Liver	I	.0	.06	10.4	.0
	II	.0	.1	6.2	.024
	III	.0	.16	8.2	.0
	IV*	.0	.09	7.5	—
Bull Testes	I	.0	.1	1.0	.0

* Rat Liver III extracts (both diced and blended) were kept at 5° for 24 hours, and analyzed a second time.

TABLE IV.
Change in Activity of Extracts upon Standing at 5°.

	Fresh		2nd day		3rd day	
Tissue	DRNase	Inh.	DRNase	Inh.	DRNase	Inh.
Lymph node metastasis	.0	2.0	15	.0	.0	.0
" " (normal)	7.0	2.0	—	—	14.0	.0
Cancer of the breast	.0	1.8	7	.0	—	—

(Results are in viscosity units.)

of the liver into "soluble", "particulate", and "nuclei" fractions appeared advisable. This was accomplished using isotonic sucrose according to the method of Schneider(9). Neither the enzyme nor the inhibitor could be shown in the "nuclei" fraction. The "particulate" fraction always represented the fraction richest in enzyme, as would be expected from the previous observation. The "soluble" fraction was most erratic, showing the predominance of either enzyme or inhibitor. This was probably due to the fluctuations in the rate of liberation of desoxyribonuclease from the "particulate" fraction into solution.

Evidence that on standing the two components are destroyed at different rates, probably as a result of proteolysis, is presented in Table IV. The "blended' extracts prepared from human tissues, after being centrifuged, were exposed to 5° for different periods of time. The inhibitor was the first to disappear. The apparent increase in the amount of enzyme which was seen after 2 days' exposure was most probably due to the removal of the masking effect of the inhibitor, since on longer exposure desoxyribonuclease also disappeared. Thus far, attempts to devise a method which would quantitatively separate the enzyme and the inhibitor have proved unsuccessful.

Summary. The reaction between desoxyribonuclease and the inhibitor for desoxyribonuclease was found to be of the reversible type, similar to that observed by Kunitz between chymotrypsin α and soybean trypsin inhibitor.

A method for the semi-quantitative evaluation of the inhibitor for desoxyribonuclease has been described. Several normal and pathological tissues were analyzed for both desoxyribonuclease and the inhibitor. Both measurements were influenced by the presence of the second component of the pair.

The inhibitor was found to be readily soluble, while desoxyribonuclease was only slowly liberated into solution from the particulate matter of the cell. Neither of the two components was resistant to the proteolytic enzymes during autolysis, the inhibitor being the least stable of the two.

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Androgenic Activity of C21 Steroids.[*] (17635)

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Previous studies by Pfiffner and North(1,2) have indicated that 17(α)-hydroxyprogesterone is an androgen and possesses an activity approximately equal to that of androsterone on the immature castrate rat seminal vesicle and prostate. When administered intramuscularly to capons this compound was less than one half as active as androsterone on the comb. These studies were done with a limited quantity of material isolated from the adrenal gland. With the availability of this and related compounds as the result of partial synthesis it has become possible to study the androgenic activity of 17(α)-hydroxyprogesterone and related compounds in more detail. This communication is concerned with the influence of 17 (α)-hydroxyprogesterone, Δ^{16}-dehydroprogesterone, Δ^{4}-pregnenetriol-17(α), 20 (β), 21-one-3-diacetate-20,21 and 17-hydroxy-11-desoxycorticosterone acetate (Compound "S" acetate, Reichstein) on the chick's comb.

Experimental. Androsterone[†] (used as the reference compound) and the 4 steroids, 17 (α)-hydroxyprogersterone,[‡] Δ^{16}-dehydroprogesterone,[§] Δ^{4}-pregnenetriol - 17(α),20(β), 21-one-3-diacetate-20,21,[‡] were dissolved in corn oil so that the daily dose was contained in 0.05 cc. The material was applied to the comb once daily for 7 days starting 2 days after the male white leghorn chicks[‖] were hatched. Twenty-four hours after the last applications comb and body weight were determined. The results are expressed as the ratio of the comb weight to body weight. In 2 instances the steroids were administered by subcutaneous injection, the daily dose being contained in 0.1 cc of corn oil. All other details were similar to that described for the direct comb application technic.

Results and conclusions. The results are tabulated in Table I. Δ^{16}-Dehydroprogesterone, applied directly to the comb, in a dosage of 500 μg, produced in 2 experiments significant increments of 97 and 124% in comb weight. A total dose of 940 μg produced a comb weight increase of 190%. By direct comb application this C21 steroid, Δ^{16}-dehydroprogesterone, was found to be about 6% as active as androsterone.

17(α)-Hydroxyprogesterone was studied by direct comb application, at the 80, 320, and 640 μg dosage levels. Neither 80 nor 640 μg produced any significant changes in comb weight, but at 320 μg a statistically significant increase of 29% was observed ($t = 3.90$). When a total dose of one mg was injected a statistically significant decrease in comb ratio of 18% ($t = 2.78$) was found.

Δ^{4}-Pregnenetriol-17(α),20(β),21-one-3 diacetate-20,21 was applied to the comb in total doses of 500 and 840 μg. Statistically significant decreases in comb ratio were observed in both instances. At the lower dose level a decrease of 26% was found and at the higher dose level a decrease of 21% was observed. The fact that local application inhibited the growth of the chick's comb speaks for direct antagonism to endogenous androgen stimula-

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[*] Supported in part by a grant (RG 1050) from the Division of Grants and Fellowships, National Institutes of Health, U. S. Public Health Service.

1. Pfiffner, J. J., and North, H. B., *J. Biol. Chem.*, 1940, v132, 459.

2. Pfiffner, J. J., and North, H. B., *J. Biol. Chem.*, 1941, v139, 855.

[†] Androsterone was kindly supplied by Ciba Pharmaceutical Products, Inc.

[‡] Δ^{4}-Pregnenediol-17(α),21 - one - 3 - acetate-21 (compound "S" acetate, Reichstein) was part of a gift from Mr. Adrian Joyce of the Glidden Co. 17(α)-Hydroxyprogesterone and Δ^{4}-pregnenetriol-17(α),20(β),21-one-3-diacetate-20,21 were purchased from the Glidden Co.

[§] Δ^{16}-Dehydroprogesterone was kindly supplied by Ayerst, McKenna, and Harrison Limited in collaboration with Syntex, S.A.

[‖] The chicks were purchased from the Kerr Chickeries, Frenchtown, N.J.

TABLE I.
Action of Various C-21 Steroids and Androsterone on Chick's Comb.

Exp. No.	Compound (Method of administration)	Total dose µg	No. of Chicks	Comb ratio* Mean S.E.	Change in comb ratio* %	"t"
1.	0	0	22	35 ± 1	—	—
	Androsterone (Application)	20	14	62 ± 5	+ 77	—
		40	15	88 ± 7	+151	—
		80	15	125 ± 10	+257	—
	17(α)-Hydroxyprogesterone (Application)	80	15	35 ± 1	0	—
		320	16	45 ± 2	+ 29	3.90
		640	15	37 ± 2	+ 6	—
	Δ^{16}-Dehydroprogesterone (Application)	500	15	69 ± 5	+ 97	
	Δ^{4}-Pregnenetriol-17(α),20(β),21-one-3-diacetate-20,21 (Application)	500	16	26 ± 2	— 26	4.23
	Δ^{4}-Pregnenediol-17 (α),21-dione-3,20-acetate-21 (Compound "S" acetate, Reichstein) (Application)	500	16	27 ± 2	— 23	3.73
2.	0	0	12	38 ± 1	—	—
	Δ^{16}-Dehydroprogesterone (Application)	500	9	85 ± 6	+124	—
		940	9	110 ± 14	+190	
	Δ^{4}-Pregnenediol-17(α),21-dione,3,20-acetate-21 (Injection)	1000	10	27 ± 1	— 29	6.36
	Δ^{4}-Pregnenetriol-17(α),20(β),21-one-3-diacetate-20,21 (Application)	840	11	30 ± 2	— 21	3.20
	17(α)-Hydroxyprogesterone (Injection)	1000	12	31 ± 2	— 18	2.78

$$\text{* Comb Ratio} = \frac{100 \times \text{Comb wt (mg)}}{\text{Body wt (g)}}$$

tion. Δ^4-Pregnenediol-17(α),21-dione-3,20-acetate-21 (compound "S" acetate, Reichstein) inhibited the growth of the comb when applied directly or when administered subcutaneously. When 500 μg was applied to the comb a decreased comb ratio of 23% ($t = 3.73$) was found, while one mg administered subcutaneously produced a decrease of 29% ($t = 6.36$).

Summary. Δ^{16}-Dehydroprogesterone exhibited androgenic activity by direct application to the chick's comb. This steroid has approximately 6% of the activity of androsterone. Δ^4-Pregnenetriol-17(α),20(β), 21-one-3-diacetate-20,21, and Δ^4-pregnenediol-17(α),21-dione-3,20-acetate-21 (compound "S" acetate, Reichstein) inhibited the growth of the comb probably by direct antagonism of endogenous androgens. 17(α)-Hydroxyprogesterone produced no consistent effect. By direct comb application 80 and 640 μg levels were without effect while the 320 μg dose produced a 29% comb ratio increment which was statistically significant. When this steroid was injected subcutaneously at a dose of 1000 μg a significant comb inhibition was observed.

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Complement-fixing and Neutralizing Antibody Studies on Humans Vaccinated Against Rabies. (17636)

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Many workers have reported that neutralizing antibodies develop in the serum of animals and of human beings treated with live or killed rabies vaccines(1-5). It also has been shown that complement-fixing antibodies develop to a high titer in rabbits, mice, guinea pigs and dogs hyperimmunized by repeated injections of living virus(6). The somewhat meager evidence to date indicates that the two antibodies are not identical. Thus Habel(7) found that monkeys developing rabies showed no complement-fixing antibodies although virus neutralizing antibodies were present. Similarly, in experiments carried out in guinea pigs, Nachtigal(8) and Bernkopf and Nachtigal(9) showed that complement-fixing antibodies reached a maximal titer about a week after the last injection, diminished to one-eighth the maximal titer after one month, and disappeared completely within three months. On the other hand, neutralizing antibodies reached maximal titers after one month and showed no decrease in titer during the subsequent three months of observation.

This communication reports on the results obtained in determining to what extent neutralizing and complement-fixing antibodies are induced in human beings vaccinated with the Semple-type, phenol-killed rabies vaccine(1).

Materials and Methods. Vaccinations. All vaccinations were made with the Semple-type, phenol and heat-killed vaccine consisting of 5% rabies-infected rabbit brain tissue prepared by the New York City Department of Health. Subcutaneous injections of 2 ml of vaccine were given each day either for 7 or for 14 consecutive days. The course of treatment was determined on the basis of the criterion set by the New York City Department of Health which stipulates that 14 injections are to be given to those individuals who are bitten by stray animals or by such animals that are known to be rabid. The course of 7 injections is given to those who have lesions above the clavicle if the animal is available for examination, and continued through the whole series of 14 injections if such an animal is proven to be rabid. No injections are given to persons with bites inflicted below the clavicle if the animal is known.

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Blood samples. The first blood sample was withdrawn immediately prior to the first injection of vaccine, the second on the day of completion of the treatment, and the subsequent samples at various intervals thereafter. Serum samples were stored in rubber-stoppered vials in the CO_2 box until used, at which time they were inactivated at 56°C for 30 minutes.

Neutralization tests. The Lederle strain of mouse-brain fixed virus was made up in a 20% suspension and serial tenfold dilutions were made from the centrifuged supernate in buffered saline solution containing 10% inactivated normal rabbit serum. Each virus dilution was mixed with an equal volume of undiluted human serum, incubated for 2 hours at 37°C, then inoculated intracerebrally into albino Swiss mice 4 to 5 weeks old. Fifty per cent lethal endpoints were determined. Inactivated and undiluted normal human serum was used for controls.

Complement-fixation tests. Mouse brain tissue infected with the Lederle strain of fixed rabies virus was used as specific antigen after being benzene extracted from the dried state by the method of DeBoer and Cox(10). Normal non-infected mouse brain tissue was similarly processed and used in all tests as control antigen. The method used was essentially the overnight ice-box technique of Kolmer and Boerner(11), as modified for use in our laboratory(10,12). The customary serum and antigen controls were included in each test. A 2 + fixation, or higher, was taken as the endpoint.

Experimental. From the total number of 2,841 individuals who presented themselves for antirabies treatment at the Manhattan Clinic between January 1, 1946, to December 31, 1948, random blood samples were taken from approximately 100 persons. Of these, serial specimens were tested from 18 individuals who had received a series of 7 injections and from 69 who had received 14 injections of vaccine. Of those who received 7 injections of vaccine, only one individual had received antirabies treatment approximately 18 months previously. Of those who received 14 injections, antirabies treatment had been received by one individual 6 months previously, and by two others 2 years previously. The 4 individuals who had had previous antirabies treatment were excluded from all calculations concerning the comparative geometric mean antibody titers.

Table I shows the comparative geometric mean of neutralizing indices and complement-fixation (C-F) titers in patients who received 7 injections of vaccine (Series A), whereas Table II shows similar data for those who received 14 injections of vaccine (Series B). It is quite apparent from the data of these two tables that both neutralizing and C-F antibodies were induced in the two groups of vaccinated individuals, but that the neutralizing antibodies reached higher levels and persisted longer than did the C-F antibodies—with the highest antibody levels being attained in the B series of patients. Thus, in the A series (Table I) both types of antibodies began to appear by the first week after treatment, and reached their maximum 4 to 5 weeks after treatment. The C-F antibodies had disappeared entirely by the 4th month, whereas the neutralizing antibodies persisted in

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TABLE I.
A Series: Course of 7 Injections of Vaccine.
Comparative Geometric Mean of Neutralizing and Complement-fixing Antibody Titers.

Blood samples	Neutralization index*	Complement-fixation titer*
Pre-treatment	2.2 (13)	.1 (16)
At completion of treatment	3.3 (14)	.1 (16)
1 week after treatment	46.7 (16)	2.9 (15)
4-5 ’’ ’’ ’’	429.5 (9)	3.4 (9)
6-7 ’’ ’’ ’’	83.9 (4)	3.0 (5)
17-25 ’’ ’’ ’’	303.4 (11)	.0 (10)
29-32 ’’ ’’ ’’	301.3 (5)	.0 (5)
33-46 ’’ ’’ ’’	91.2 (5)	.0 (5)

* The figures in parentheses represent the number of serum specimens comprising the sample used for calculation of geometric mean titer.

TABLE II.
B Series: Course of 14 Injections of Vaccine.
Comparative Geometric Mean of Neutralizing and Complement-fixing Antibody Titers.

Blood sample	Neutralization index*	Complement-fixation titer*
Pre-treatment	3.8 (50)	0.1 (61)
At completion of treatment	175.8 (66)	2.6 (63)
1 week after treatment	5445 (64)	6.1 (59)
4-5 ’’ ’’ ’’	7780 (45)	4.6 (44)
6-7 ’’ ’’ ’’	2576 (17)	4.0 (23)
17-25 ’’ ’’ ’’	981.7 (56)	1.4 (56)
29-32 ’’ ’’ ’’	576.8 (36)	1.0 (36)
33-46 ’’ ’’ ’’	914.1 (13)	1.1 (14)

* Same as in Table I.

TABLE III.
Comparative Antibody Titers of 4 Individuals Who Had Had Previous Antirabies Treatment.

	Neutralization index Patient No.				Complement-fixation Patient No.			
Blood sample	1	2	3	4	1	2	3	4
Pre-treatment	794	1580	100	5	0	0	0	0
At completion of treatment	7940	>31600	>31600	7940	32	32	128	8
1 week after treatment	>25100	>31600	>31600	7940	32	32	32	4
4-5 ’’ ’’ ’’	—	20000	>63100	—	—	32	16	—
6-7 ’’ ’’ ’’	>31600	—	—	—	8	—	—	—
17-25 ’’ ’’ ’’	20000	10000	>15800	—	4	8	8	—
29-32 ’’ ’’ ’’	—	—	>31600	—	—	—	4	—
33-46 ’’ ’’ ’’	—	1580	—	—	—	8	—	—

Patient No. 1: Received 7 inj. in present series; had rabies vaccine 18 mo. before.
Patient No. 2: Received 14 inj. in present series; had rabies vaccine 6 mo. before.
Patients Nos. 3, 4: Received 14 inj. each in present series; had rabies vaccine 2 years before.

significant titer through the 8th to 12th month. The same general picture was true also for the B series (Table II) although here the antibodies began to be apparent at time of completion of treatment. Furthermore, the maximal level of the neutralizing antibodies of the B series was approximately 18 times that of the A series.

Table III shows the results obtained with serum specimens from the 4 individuals who had had antirabies vaccine 6 months to 2 years prior to the present course of treatment. Patient No. 1 received 7 injections of vaccine during the present series, while the remainder all received 14. Two rather interesting points are apparent in the table. First, it is seen that 3 of the 4 pre-treatment serums showed appreciable amounts of neutralizing substances with the titers apparently being inversely proportional to the time interval elapsing after vaccination. This, of course, indicates how antibodies of this type persist for fairly long periods of time in the blood. Secondly, it is seen that quite a marked antibody rise or "booster" effect resulted following the second series of vaccine injections.

Discussion. The results shown here with serum specimens obtained from individuals who had received antirabies treatment are in full accord with those of Habel(7), Nachtigal(8), and Bernkopf and Nachtigal(9) who carried out similar tests on somewhat more limited number of serum specimens from vaccinated animals and showed that the virus neutralizing antibodies are quite distinct from the complement-fixing antibodies on the basis of the marked differences noted in their comparative titration endpoints and time intervals of maintenance. No attempt has been made in this study to determine the nature or properties of the 2 types of antibodies, nor is it intended to imply that there is any correlative relationship between either or both of these antibodies and the degree of immunity attained by the vaccinated host. In this connection it is of interest to mention briefly the work of others in this field. Nachtigal(8) reports that 2 independent and different antigens are involved in eliciting neutralizing and complement-fixing antibodies. This was proven by ultracentrifugation experiments with infected mouse-brain suspensions and comparison of the infective and the complement-fixing titers of the sediment and the supernatant fluid. By use of the ultracentrifuge it was possible to sediment the bulk of the virus without decreasing the original complement-fixing titer of the supernate. The virus-containing sediment did not fix complement at all. Thus the rabies antigen specifically involved in the production of complement-fixing antibodies is not pathogenic, is an entity separate from the infective virus itself, and is apparently of smaller particle size. This of course indicates that rabies has a "soluble" antigen similar to those known to exist in many other virus infections.

Finally it is evident from the literature that there is divergence of opinion as to whether serum neutralizing antibodies indicate resistance of the host to infection or not. However, the great bulk of evidence indicates that there is no essential or necessary parallelism between the neutralizing antibody titer of the serum and the degree of resistance of the host to infection (see 4,5,13,14,15,16). The studies of Kubes and Gallia(5) are worthy of mention here. These workers, while agreeing that the serum neutralization test does not give an accurate index of the immunity status of the host, claim that the brain-tissue neutralization test carried out with brain tissue suspensions of immunized mice gives a true picture of the antirabies immunity of the organism. While this type of study is worthwhile and feasible from the laboratory point of view, obviously it could not be carried out with human beings.

Summary. 1. Blood samples taken prior to vaccination and at rather frequent intervals after completion of antirabies vaccination were tested from 18 individuals who received 7 injections of vaccine and from 69 individuals who received 14 injections of vaccine.

2. Both neutralizing and complement-fixing antibodies were induced in the two groups of vaccinated individuals, but in all instances the neutralizing index reached higher levels and persisted longer than did the complement-fixation titer. The highest antibody levels were attained in those patients who received the greater number of vaccine injections.

3. Four individuals had had antirabies vaccine 6 months to 2 years previously. Three of the 4 still showed an appreciable neutralizing index prior to being revaccinated, and all 4 showed a marked antibody rise or "booster" effect after the second series of vaccine injections.

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Dialyzable Factor in Allantoic Fluid Affecting Virus Hemagglutination-Inhibition Reactions.* (17637)

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(Introduced by J. W. Beard.)
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The virus of swine influenza, purified by centrifugal procedures from the allantoic fluid of infected chick embryos(1), shares with human influenza viruses the capacity to agglutinate chicken red blood cells(2,3). The agglutination reaction is inhibited strongly by immune swine serum and weakly by normal serum, egg-white(4), and cow's milk(5). However, inhibition by the latter fluids, especially egg-white and milk, is considerably enhanced if purified virus heated at 53°C for 30 minutes is employed as the agglutinating agent(4,5). This increased susceptibility to inhibition, first described by Francis(6) for heated influenza virus B, has been attributed to an enzyme-like capacity of unheated virus to destroy inhibitor and to the loss of destructive activity on heating (7-9) (cf., however(10)).

Recently, it was found that swine influenza virus *in allantoic fluid* fails to develop on heating the high inhibition susceptibility characteristic of heated purified virus. Experiments carried out to elucidate this phenomenon, which would appear to have significance for numerous investigations employing unpurified virus, are described in the present report.

Materials and methods. Several sterile pools of normal allantoic fluid and of fluid infected with swine influenza virus (Shope's strain 15) or influenza virus A (PR8 strain) were employed. Embryonated 11-day eggs, obtained from a local hatchery, were inoculated with swine or PR8 virus and harvested as previously described(1,11) after 40 hours at 35°C (swine) or 37°C (PR8). Normal fluid was obtained from similar eggs after 40 hours at 35°C. The fluids were stored at 4°C without preservative. Before use, they were centrifuged at low speed, if necessary, to remove precipitates. Swine influenza virus was purified from a portion of one of the pools (Pool 1) by routine centrifugal procedures(12) with the modification that the pellets of the final cycle were taken up in 0.06 M phosphate buffer at pH 7.3 instead of Ringer solution. The product, labeled SF32-PB and stored at 0.217 mg N per ml at 4°C, possessed about 25,000 chick cell hemagglutinating doses per mg N and a 50% endpoint infectious unit of $10^{-13.7}$ g N on 10/17/49, 17 days after the purification was completed.

Hemagglutinative activity was determined by the method of Hirst(13), as modified in previous studies from this laboratory(14).

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TABLE I.
Comparison of Purified (SF32-PB) and Unpurified Swine Influenza Virus with Respect to Development of Susceptibility to Egg-White Inhibition on Heating.

Virus	Conditions of heating				Inhibition titer[§]
	HD per ml	Diluent	pH[‡]	Min. at 53°C	
SF32-PB				0	20
,,	24	Buff. saline[*]	7.3	30	49,000
,,	24	Phos. buffer[†]	7.3	30	58,000
Pool 1				0	<20
,,	96	None	8.5	30	50
,,	32	Buff. saline	8.2	15	64
,,	32	,, ,,	8.2	30	80
,,	32	,, ,,	8.2	60	101
,,	8	,, ,,	7.6	30	115
Pool 2				0	<20
,,	72	None	8.1	30	92
,,	24	Phos. buffer	7.4	30	113
,,	8	,, ,,	7.3	30	450

[*] 0.81% NaCl and 0.005 M phosphate at pH 7.3.
[†] 0.06 M phosphate buffer at pH 7.3.
[‡] By glass electrode.
[§] Determined against 4 HD (hemagglutinating doses).

One *hemagglutinating dose* (HD) of virus is the amount required to give the standard two-plus (++) endpoint of agglutination with washed chicken red blood cells. *Heated virus* was prepared by immersing a rubber-stoppered test tube, containing a suitable dilution of virus, in a water thermostat at 53.0 ± 0.1°C. After a suitable period, usually 30 minutes, the tube was cooled in running tap water, and the virus was used immediately.

Inhibition titrations, employing a 20% solution of egg-white (EW) in buffered saline, preserved with 0.02% Merthiolate (Lilly), were carried out by the constant virus-varying inhibitor method(15). The *inhibition titer,* determined against 3 to 4 HD of virus, is expressed as the reciprocal of the final dilution of EW in the reaction mixture giving two-plus hemagglutination. Unless otherwise specified, the diluent was *buffered saline,* which consisted of 0.81% NaCl and 0.005 M phosphate at pH 7.3. A stronger buffer, consisting of 0.06 M phosphate at pH 7.3, was occasionally employed. Borate buffer at pH 7.4 was prepared according to Palitzsch, as given by Clark(16). All chemicals were of the reagent grade. Other details are given with the description of the experiments.

Experimental. Comparison of purified and unpurified virus. Samples of allantoic fluid infected with swine influenza virus (Pool 1, 96 HD per ml, and Pool 2, 72 HD per ml) and of virus (SF32-PB) purified from Pool 1 were heated at 53°C as shown in Table I and tested for susceptibility to EW inhibition by the routine method. The purified virus behaved typically(4) in developing a high susceptibility to inhibition on heating. The unpurified virus, on the other hand, developed only a low susceptibility. The inhibition titer against the latter was slightly improved by prolonging the period of heating to 60 minutes or by diluting the virus before heating (Table I). No greater increase in inhibition titer was obtained by heating unpurified virus at 56°C for 10 minutes; the rapid decrease in the hemagglutinative activity of the virus at the higher temperature precluded the use of longer periods of heating.

Adequate control of pH during heating was obtained by the use of 0.06 M phosphate buffer at pH 7.3 as diluent. The general similarity of the results obtained with this diluent and with the weaker buffered saline indicates

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TABLE II.
Effect of Heat on Dialyzed and Non-dialyzed Swine Influenza Virus in Allantoic Fluid (Pool 2). Reversibility of the Effect of Dialysis.

Mixture		Further treatment	Before titration, Mixture diluted 3-fold§ with	Inhibition titer
1 Vol.	2 Vol.			
Pool 2	Phos. buffer	None	Phos. buffer	<20
” ”	” ”	Heated‡	” ”	101
” ” dialyzed*	” ”	None	” ”	<20
” ” ”	” ”	Heated	” ”	64,000
” ” ”	Semi-dialysate†	None	” ”	<20
” ” ”	” ”	Heated	” ”	127
” ” ”	Phos. buffer	”	1:3 Semi-dialysate	45,000
” ” ”	” ”	None	1:3 Heated‡ semi-dialysate	<20
” ” ”	” ”	Heated	1:3 ” ”	74,000

* Overnight in the cold against 100 volumes 0.06 M phosphate buffer at pH 7.3.
† Prepared by dialysis of Pool 2 against 1 volume of phosphate buffer.
‡ 30 min. at 53°C.
§ To give 4 HD in 0.5 ml. No significant decrease in hemagglutinative activity occurred during either dialysis or heating.

that pH is not a highly significant variable under the conditions of the experiments.

Effect of dialysis. The difference in behavior of purified and unpurified virus may be ascribed (a) to a difference in the properties of the virus particles or (b) to an extrinsic factor other than pH. Since the purified virus was obtained by relatively mild procedures, the latter alternative appeared the most likely explanation. Accordingly, virus-infected allantoic fluid was dialyzed in Visking casing overnight in the cold against 100 volumes of 0.06 M phosphate buffer at pH 7.3. A second sample was dialyzed against one volume of phosphate buffer under similar conditions in order to provide the dialyzable components of infected allantoic fluid in relatively high concentration; dialysate obtained in this way is referred to as *semi-dialysate*. In this experiment, phosphate buffer was used as diluent up to the point of the inhibition titrations.

Tests showed (Table II) that the thoroughly dialyzed virus developed on heating a high susceptibility to EW inhibition, comparable to that observed with centrifugally purified virus. Moreover, the effect of dialysis could be reversed by adding semi-dialysate to thoroughly dialyzed virus before heating; semi-dialysate, heated or not heated, added to heated dialyzed virus was without effect.

It appears, therefore, that the failure of unpurified virus to develop a high susceptibility to EW inhibition on heating may be ascribed to the influence of a dialyzable factor (or factors) in infected allantoic fluid. The results (Table II) show that this factor exerts its effect during the heating of the virus and not during the subsequent inhibition titration. The sensitivity of purified virus to heat (Table I) is readily explained on the reasonable assumption that the dialyzable factor, initially present in the infected allantoic fluid, is lost during the process of purification of the virus.

Occurrence of dialyzable factor in normal allantoic fluid. The dialyzable factor was found also in normal allantoic fluid. Furthermore, semi-dialysate from normal fluid was effective against virus purified by centrifugation (SF32-PB) as well as against dialyzed virus, as illustrated in Table III. These experiments followed the lines of the experiment recorded in Table II (above) and will not be described in detail.

The results indicate that the dialyzable factor is a normal constituent of allantoic fluid, independent of infection by influenza virus. The factor seems to be unrelated to the normal allantoic inhibitor of influenza virus hemagglutination(17) since (a) the normal inhibitor is not dialyzable and (b) thoroughly dialyzed normal allantoic fluid, although retaining inhibitory activity against

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TABLE III.
Effect of Dialysate from Normal Allantoic Fluid on Dialyzed Virus and Purified Virus (SF32-PB).

Virus	Treatment	Inhibition titer
Pool 1*	None	<20
Pool 1	Heated‡	200
Pool 1, dialyzed	None	<20
” ”	Heated	76,000
” ”	” with normal semi-dialysate	160
SF32-PB†	None	20
”	Heated	58,000
”	” with normal semi-dialysate	230

* Swine influenza virus in allantoic fluid.
† Diluted to approximately the same hemagglutinative activity as Pool 1.
‡ 30 min. at 53°C at a concentration of about 20 HD per ml.

TABLE IV.
Inhibitor Susceptibility of Thoroughly Dialyzed Swine Influenza Virus in Allantoic Fluid (Pool 3) Heated in the Presence of Ca^{++} or Mg^{++}.

2 vol. 1:3 dialyzed virus + 1 vol.	Further treatment	Inhibition titer
Buffered saline	None	<20
” ”	53°C, 30 min.	68,000
Semi-dialysate*	” ”	210
9×10^{-4} M $CaCl_2$†	” ”	53
3×10^{-4} M ”	” ”	210
9×10^{-5} M ”	” ”	2,100
3×10^{-5} M ”	” ”	41,000
3×10^{-3} to 3×10^{-5} M $MgCl_2$	” ”	68,000

* Obtained by dialysis of Pool 3 against 1 vol. of buffered saline.
† 3×10^{-3} M $CaCl_2$ in buffered saline formed a precipitate and was not tested.
No precipitation occurred in the other solutions; nor did the hemagglutinative activity decrease significantly during heating.

virus hemagglutination, does not exert the effect described above.

Identification of the dialyzable factor with Ca^{++}. Recent reports(18-23) have stressed the significance of Ca^{++} and calcium deionizing agents for certain *in vitro* properties of influenza viruses. Accordingly, Ca^{++}, which is reported (24) to be present in allantoic fluid in a concentration of about 0.019% (about 4.8×10^{-3} M), was tested for its capacity to substitute for the dialyzable factor. Mixtures of unpurified swine influenza virus (Pool 3; 80 HD per ml), thoroughly dialyzed against buffered saline, and of graded amounts of $CaCl_2$ in buffered saline were heated as usual for 30 minutes at 53°C. The products were then tested in the usual manner for susceptibility to inhibition by EW. Buffered saline, although containing phosphate and allowing the possibility of precipitation of the added calcium, was employed as diluent since phosphate was present in all the previous experiments.

The results, presented in Table IV, show that Ca^{++} is indeed capable of suppressing the development of inhibitor sensitivity by virus on heating. A highly significant effect was observed when virus was heated in the presence of 3×10^{-5} M $CaCl_2$, and a slight,

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TABLE V.
Effect of Citrate, Oxalate, and Phosphate on the Development of Inhibitor Sensitivity by Non-dialyzed Swine Influenza Virus in Allantoic Fluid (Pool 4) on Heating (53°C, 30 Min.).

1:5 Pool 4* plus 0.2 vol.	pH before heating	pH after heating and 2-fold dil.† with 0.85% NaCl	Inhibition titer
.85% NaCl	7.6	7.7 (7.7)‡	140 (<20)‡
.128 M $Na_3C_6H_5O_7$	7.7	7.8	82,000
.032 ”		7.7	34,000
.008 ”		7.7	240
.002 ”		7.7	200
.192 $Na_2C_2O_4$	7.6	7.7	82,000
.048 ”		7.7	82,000
.012 ”		7.7	200
.003 ”		7.7	200
.192 Na_2HPO_4	7.7	7.8	900
.048 ”		7.8	180
.012 ”		7.7	180
.003 ”		7.7	140
[Dialyzed control §]	7.6	7.7	82,000

* Diluted with borate buffer at pH 7.4.

† To give 4 HD in 0.5 ml. No significant decrease in hemagglutinative activity of any of the mixtures occurred during heating.

‡ Data in parentheses refer to unheated control with same composition.

§ Pool 4 dialyzed against 100 volumes buffered saline, diluted 5-fold with borate buffer, mixed with 0.2 volume 0.85% NaCl, heated, etc.

possibly insignificant effect was observed at 10^{-5} M. In the same experiment semi-dialysate was found equivalent to a 3×10^{-4} M solution of $CaCl_2$. In view of the presence of phosphate in the mixtures, these values are regarded to indicate only the maximal quantity of available Ca^{++}. In striking contrast with Ca^{++}, Mg^{++}, also present in allantoic fluid(24), was ineffective in substituting for semi-dialysate.

In other experiments undialyzed virus (Pool 4; 96 HD per ml) was heated in the presence of various amounts of citrate ($Na_3C_6H_5O_7 \cdot 5\frac{1}{2} H_2O$), oxalate ($Na_2C_2O_4$), and phosphate ($Na_2HPO_4$), as shown in Table V. The solutions at the highest concentrations shown were prepared in distilled water; the first 4-fold dilution of each was made with water in order to bring the ionic strength to a suitable level, and the subsequent dilutions were made with 0.85% NaCl. The mixtures were allowed to stand at room temperature about 10 minutes before heating. Within 5 minutes after preparation the mixtures containing oxalate in the two highest concentrations developed a faint turbidity, which did not increase appreciably during subsequent heating.

Both citrate and oxalate were effective in increasing the development of inhibitor sensitivity by virus on heating; phosphate was relatively ineffective. The maximal titer attained with virus treated with citrate or oxalate was identical with the titer attained with virus subjected to dialysis. The data (Table V) suggest that in certain equivalent concentrations oxalate might be more effective than citrate, a point which has been carefully checked for the conditions of the present experiments; however, it would be premature to conclude that this relation would hold under other conditions, in view of the high sensitivity of the system to the concentration of Ca^{++} (Table IV). These results with deionizing agents further support the conclusion that Ca^{++} is the agent in allantoic fluid responsible for the effects noted in Tables I to III.

Experiments with Human Influenza Virus A (PR8 Strain). The possibility that the foregoing results with swine influenza virus might be of general significance for influenza viruses was examined in experiments with allantoic fluid infected with the PR8 strain of human influenza virus A. It was found that this virus, in contrast with the swine virus, became highly susceptible to inhibition

TABLE VI.

Comparison of Swine Influenza Virus (Pool 3) and Human Influenza Virus A (PR8 Strain).

Mixture*			
1 volume diluted dialyzed† virus	1 vol.	Further treatment	Inhibition titer
Swine influenza	Buffered saline	None	<20
,, ,,	,, ,,	53°C, 30 min.	68,000
,, ,,	Semi-dialysate (Swine)‡	,, ,,	180
,, ,,	,, (PR8)‡	,, ,,	130
PR8	Buffered saline	None	<20
,,	,, ,,	53°C, 30 min.	17,000§
,,	Semi-dialysate (Swine)	,, ,,	14,000
,,	,, (PR8)	,, ,,	17,000

* All mixtures contained 16 HD per ml. The PR8 stock had about 170 HD per ml.

† Against 100 volumes of buffered saline.

‡ Prepared by dialysis against 1 volume of buffered saline. The pH was: Swine, 8.3; PR8, 8.1.

§ Identical titers were obtained with non-dialyzed virus heated 30 min. at 53°C and with dialyzed and non-dialyzed virus heated 30 min. at 56°C. In contrast with the swine virus, PR8 virus, whether dialyzed or not, lost 40 to 50% of its hemagglutinative activity on heating for 30 min. at 53°C or 56°C. Suitable adjustments were made prior to the inhibition tests.

by EW on heating and that dialysis prior to heating was without consequence.† The question therefore arose whether the dialyzable factor, present in normal and in swine-infected allantoic fluid, might be absent from PR8-infected fluid. Accordingly, semi-dialysate from each kind of virus-infected fluid was tested with both the homologous and the heterologous dialyzed viruses. As seen in Table VI, both semi-dialysates were effective against swine influenza virus and neither was effective against PR8 virus. In other experiments PR8 virus in allantoic fluid was heated in the presence of $CaCl_2$, added to give concentrations ranging from 10^{-5} to 3×10^{-3} M during heating. A slight effect was observed at the highest concentration only. It would appear, therefore, that PR8 and swine influenza viruses differ remarkably in their sensitivity to Ca^{++}.

Discussion. The primary object of the present investigation was to account for the difference in behavior of purified and unpurified swine influenza virus preparations heated for 30 minutes at 53°C. The titer of egg-white inhibitory activity against hemagglutination by heated purified virus is about 50,000. Against heated unpurified virus (virus in allantoic fluid) the titer is about 100. Against unheated virus of either sort the titer is 20 or less. The experimental results indicate that the difference between the two heated viruses is attributable to the calcium naturally occurring in the unpurified virus preparation. Ionized calcium, in low concentration depresses the development of inhibitor sensitivity by swine influenza virus on heating. The calcium may be removed by dialysis or neutralized by citrate or oxalate; phosphate is considerably less effective. Unpurified virus treated in these ways develops a high susceptibility to inhibition by egg-white on heating. In similar experiments human influenza virus A (PR8 strain) showed very little sensitivity to calcium. Burnet and his collaborators have shown that added calcium depresses the thermal development of inhibitor sensitivity by Lee virus(19) as well as the thermal inactivation of the receptor-destroying enzyme (RDE) of the cholera vibrio(25). The enzymatic activity of both of these agents against a mucoid inhibitor occurring in ovarian cyst fluid is enhanced by calcium and depressed by calcium de-ionizing agents(19,22,25; *cf.* (18)). The activity of the de-ionizing agents decreases along the series: hexametaphosphate, citrate, oxalate, phosphate, and fluoride(22). Stone(21), working with the cyst mucoid inhibitor, has reported that unpurified swine influenza virus becomes highly susceptible to inhibition when heated at pH 8.5 for 2 hours at 37°C in borate buffer containing citrate. We have confirmed this observation only for dialyzed virus(26). Non-dialyzed virus does not develop high susceptibility to egg-white inhibition under these conditions; nor does dialyzed virus in the absence of citrate. No previous comparison of dialyzed and non-dialyzed virus with respect to development of inhibitor sensitivity on heating seems to have been made.‡ Several investigators have reported that influenza viruses in allantoic fluid differ in the extent to which they develop inhibitor sensitivity on heating(6,10,21,27,28). In particular, Bernkopf(28), employing egg-white as inhibitor, found that the PR8 virus was essentially unaffected by heat while the swine virus developed a high sensitivity to inhibitor, in contrast with the present results (Table VI).§ Hardy and Horsfall(29), however, reported a high inhibitory effect of egg-white against heated PR8 virus. These conflicting observations, while attributable to unrecognized factors, emphasize the necessity of explicit definition of the conditions under which a virus is heated. The importance of naturally occurring calcium for the properties of one virus and the lack of importance for another suggest a rational explanation for the variation among influenza viruses noted above and warn against the incautious use of unpurified virus in experiments of this sort. The mechanism by which calcium exerts its effect remains to be elucidated.

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† Similar results were obtained in recent experiments with allantoic fluid infected with influenza virus B (Lee strain).

25. Burnet, F. M., and Stone, J. D., *Austr. J. Exp. Biol. and Med. Sci.*, 1947, v25, 227.

Summary. The difference between purified and unpurified swine influenza virus with respect to development of inhibitor sensitivity on heating is ascribed to a factor, identified as calcium, occurring in normal and virus-infected allantoic fluids. The factor, which is lost, or considerably diminished, during purification, may be removed also by dialysis or neutralized by citrate and oxalate. Calcium, in low concentration, depresses the development of inhibitor sensitivity by swine influenza virus on heating; magnesium is ineffective. In contrast with the swine virus, influenza virus A (PR8 strain) is relatively insensitive to calcium. This difference between two viruses suggests a possible explanation for the generally observed variation among influenza viruses with respect to development of inhibitor sensitivity on heating.

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26. Lanni, Y. T., and Lanni, F., unpublished experiments.

‡ In a paper appearing after the present work was completed, Stone (*Austr. J. Exp. Biol and Med. Sci.*, 1949, v27, 337) reports that Lee virus, dialyzed against tap water, develops inhibitor sensitivity when incubated for 18 hours at 37°C in borate buffer (pH 8.5) containing citrate; this treatment was relatively ineffective against non-dialyzed virus.

27. Burnet, F. M., *Austr. J. Exp. Biol. and Med. Sci.*, 1948, v26, 371.

28. Bernkopf, H., PROC. SOC. EXP. BIOL. AND MED., 1949, v71, 160.

§ The identity of the virus strains studied here was confirmed by means of hemagglutination-inhibition tests with specific immune sera. We are indebted to Dr. Fred Wrights for the PR8 antiserum.

29. Hardy, P. H., Jr., and Horsfall, F. L., Jr., *J. Exp. Med.*, 1948, v88, 463.

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Anesthetic Properties of Procaine Amide (Pronestyl) and Other Amide Analogues of Local Anesthetic Compounds.* (17638)

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The recent use of N-2-diethylaminoethyl para-amino benzamide HCl in the control of cardiac arrhythmias(1) aroused interest in its anesthetic properties. This substance is the amide analogue of procaine[†], its chemical relationship to the parent substance is shown below. No investigation of the anesthetic properties of amine compounds of this type appears to have been previously reported, though, it will be recalled, that anesthetics have been developed which contain one such chain in addition to an alkoxy chain, attached to a quinoline nucleus, as for example Dibucaine (Nupercaine); and several carbanilates having anesthetic properties have previously been investigated(2,3). Preliminary experiments performed on the isolated frog sciatic nerve by techniques previously described by us(4) indicated that procaine amide was relatively inert. It was found, however, that upon intracutaneous or subcutaneous injection in man that this compound caused prompt anesthesia, the duration of which approximated that found for procaine. Accordingly it was decided to investigate these somewhat unusual pharmacological properties of procaine amide in greater detail and to determine its anesthetic index. During the course of these experiments it was decided also to compare the anesthetic properties of Beta-diethylamino-4-ethoxy benzoate (Intracaine) and N- [2-(N-ethyl-N-2-hydroxyethyl)aminoethyl] -p-butoxybenzoate HCl (Compound No. 3) together with their corresponding amide analogues. This paper contains the data obtained from these investigations. The structures of the compounds examined are presented in Table I.

Toxicity. The M.L.D.$_{50}$ for each of the 6 compounds was determined upon mice by the method adopted some years ago in this laboratory(5) and will not again be described. Table II contains the mouse M.L.D.$_{50}$ for the compounds under study. It should be noted that the toxicities of the amides do not bear a constant relationship to the toxicities of the parent substances. It is interesting to note the low toxicity of the amide analogue of Compound No. 3.

Anesthetic Potency. In vitro tests upon frogs' sciatic nerves showed that procaine, Intracaine and Compound No. 3 caused prompt interruption of conduction, whereas the corresponding amide analogues of these compounds had little or no effect on the opposite sciatic nerve of the same animal. When tested by intracutaneous and subcutaneous injections in man all the six compounds caused anesthesia and it is noteworthy that the amide analogue of Compound No. 3 was exceedingly rapid in action and caused no initial pain upon injection. Typical data from these experiments are summarized in Table III. *In vivo* tests on rats anesthetized with sodium pentothal showed that these three amide analogues produced little or no block compared with their parent substances when applied to undamaged sciatic nerves.

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* This work was supported in part by a grant from E. R. Squibb and Sons.

1. Mark, Berlin, Kayden, Rovenstine, Steele, and Brodie, *Proc. Am. Soc. for Pharm. and Exp. Therap.*, November, 1949.

[†] This compound is marketed under the trade name of Pronestyl.

2. McIntyre and Sievers, *J. Pharm. and Exp. Therap.*, 1938, v63, 369.

3. Sievers and McIntyre, *J. Pharm. and Exp. Therap.*, 1938, v62, 252.

4. Bennett, Wagner, and McIntyre, *J. Pharm. and Exp. Therap.*, 1942, v75, 125.

5. McIntyre and Sievers, *J. Pharm. and Exp. Therap.*, 1937, v61, 107.

6. Sievers and McIntyre, *J. Pharm. and Exp. Therap.*, 1937, v59, 90.

7. *Pitkin's Conduction Anesthesia*, Lippincott, Philadelphia, 1946, 232.

TABLE I.
Names and Structures of Anesthetic Compounds.

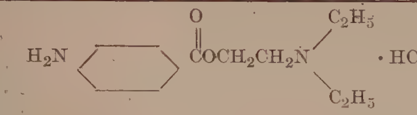
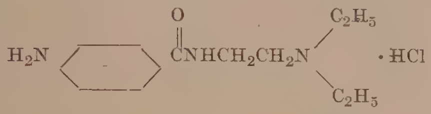
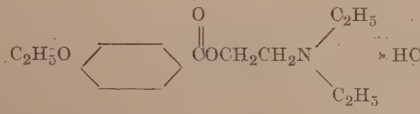
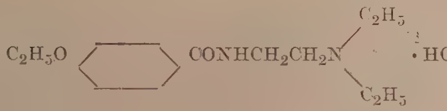
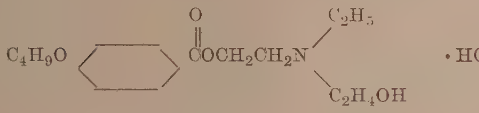
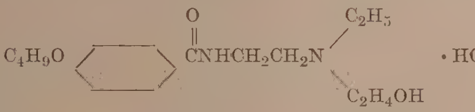
Procaine	
Procaine amide	
Intracaine	
Intracaine amide	
Compound No. 3	
Compound No. 3 amide	

TABLE II.
M.L.D.$_{50}$ and Toxicity Ratios.

Compound	M.L.D.$_{50}$ mouse,* mg/kg	M.L.D. procaine / M.L.D. compound
Procaine HCl	600 ± 20	1.00
Procaine amide HCl	800 ± 30	0.75
Intracaine HCl	430 ± 15	1.40
Intracaine amide HCl	565 ± 40	1.06
	450 ± 20†	1.32
Compound No. 3	320 ± 20	1.87
Compound No. 3 amide	900 ± 100	0.67

* The mice used in these toxicity tests were larger and less resistant to procaine than those generally used in this laboratory.

† The discrepancy is attributed to the high ambient temperature(6).

Because these anesthetics and their amide analogues differed in their actions upon intact sciatic nerves it was decided to determine their partition coefficients between frog Ringer's solution and chloroform. In preliminary experiments equimolar solutions of each of the six compounds were prepared and equal volumes of each were equilibrated with the same volume of chloroform. The Ringer's solution and chloroform were then analysed for nitrogen. However, it was found that solutions of Compound No. 3 in Ringer's solution became turbid at pH higher than 6.6, and it was decided to prepare isotonic saline solutions buffered to pH 6.6 for all the six compounds and to equilibrate with chloro-

TABLE III.
Anesthetic Potency.*

	Frog Sciatic Nerve *in Vitro*		Tests on Man (Intracutaneous)					
			Duration in min.†					
Compound	Conc. in frog Ringer's sol., mMol	Time in min. to block	Range	Mean	Min. effect conc., %	Initial pain in inj.	Erythema	Anesthetic index‡
Procaine HCl	10	5.3	13-18	15	1/40	++	+	1.00
Procaine amide HCl	10	No block in 30 min.	13-21	17	1/40	+++	+++	1.32
	90	,, ,, ,, ,, ,,						
Intracaine HCl	1	9	21-32	25	1/80	+	—	1.42
,, amide HCl	1	Slight block in 30 min.	24-36	27	1/80	+	++	1.70
Compound No. 3	0.2	11	28-56	37	1/160	++	++	2.14
,, No. 3 amide	0.2	26	31-60	35	1/160	0	0	5.96

* A 0.5% strength in isotonic saline buffer in all experiments.

† A solution of compound No. 3 cannot be buffered to a physiological pH without becoming turbid.

‡ $\frac{\text{Anesthetic potency of procaine}}{\text{Anesthetic potency of compound}} \div \frac{\text{M.L.D, compound}(7)}{\text{M.L.D. procaine}}$

TABLE IV.*
Distribution of Anesthetic Hydrochloride Salts Between Water and Chloroform.

	mMols per liter			mMols per liter		
Compound (HCl salts)	Ringer's solution	Chloroform	Ratio†	Saline buffer pH 6.6	Chloroform	Ratio†
Procaine	9.0	1.70	5.30	8.0	9.9	0.81
,, amide	10.0	0.43	23.30	22.0	0.8	24.70
Intracaine	2.9	8.70	0.30	2.4	19.5	0.12
,, amide	9.57	0.12	80.00	4.5	16.0	0.28
Compound No. 3	6.90	11.00	0.62	0.0	18.4	0.00
Compound No. 3 amide	6.18	6.18	1.00	1.5	16.0	10.61

* The authors are indebted to Dr. Fred Humoller and Miss Barbara Griswold, of this department, who kindly made the analyses for nitrogen and calculated the data for this table.

$$\dagger\text{Ratio} = \frac{\text{Millimols in } H_2O}{\text{Millimols in } CHCl_3}$$

form and again analyse for nitrogen. The results of the two series are presented in Table IV.

Discussion. Examination of the two sets of ratios obtained reveals significant differences in distribution of the compounds between the aqueous and chloroform phases. In both series the amides are less soluble in chloroform than are their parent substances. It seems probable that these differences in solubility are at least in part responsible for the much greater anesthetic activity of the parent substances, upon intact nerve trunks, compared with the relatively weak activity of their amide analogues. Presumably the lipid of the nerve sheath is penetrated relatively slowly by the amides.

Conclusions. 1. The anesthetic potencies of three anesthetics and their amide analogues have been determined and compared.

2. The amide analogues are relatively ineffective as anesthetics upon intact nerve trunks.

3. The partition coefficients between Ringer's solution and chloroform and between buffered isotonic saline of these anesthetics and their amide analogues have been determined.

4. The differences in partition coefficients between these anesthetics and their amide analogues and their pharmacological actions have been discussed.

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Application of Ion Exchange Resins to the Purification of Certain Viruses.
(17639)

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The application of ion exchange resins to the separation of closely related chemical substances by adsorption or exchange of ions(1) suggested a possible means of separating viruses from the host tissues upon which they are cultivated. Since virus particles and many host tissue components may be selectively adsorbed by a number of common adsorbents depending upon the pH of the suspending medium, their separation might be effected by passage of an infected tissue suspension through a column of ion exchange resin.

Methods. Amberlite cation exchange resin, XE-64*, between 140 and 180 mesh was used. The resin was placed in a beaker fitted with a mechanical stirrer and converted from the acid to the sodium form with 4% NaOH. After thorough washing with distilled water, the resin was then converted to a mixed sodium-acid form by the gradual addition of concentrated HCl to the resin suspension until the pH of the supernatant was at the desired point between 5.8 and 8.8. Pyrex glass chromatographic columns were then charged with the resin which was supported in the columns on coarse sand and glass wool layered over perforated glass plates. The amount of wet resin employed was twice the volume of virus suspension to be purified. The charged columns were rinsed with distilled water, drained, sterilized in an autoclave and chilled before use.

The viruses used in these experiments were: Japanese encephalitis (Nakayama strain), Eastern equine encephalomyelitis (strain 86), Western equine encephalomyelitis (Rockefeller strain), and rabies virus (CVS strain) obtained from the Virus and Rickettsiae Strain Collection at the Army Medical Department Research and Graduate School. The tissue suspensions were prepared from normal and infected chick embryos and mouse brains homogenized in a Waring Blendor with sufficient phosphate-buffered physiological saline (pH 7.7) to make 5 to 20% suspensions. Following centrifugation at 2000 r.p.m. for 10 minutes to remove coarse tissue particles, aliquots of the cleared suspensions were pipetted into the resin columns. With the aid of vacuum the fluid was pulled through the columns in 15 to 30 minutes. Samples were saved from the original centrifuged suspensions and from the percolates for titration of virus infectivity and for determination of total nitrogen by the micro-Kjeldahl method. The infectivity of preparations was determined by intracerebral titrations using groups of

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1. Applezweig, N., *Annals N. Y. Acad. Sci.*, 1948, XLIX, Art. 2, 295.

* Manufactured by Rohm and Haas Co., Philadelphia, Pa.

TABLE I.
pH and Total Nitrogen Content of Tissue Suspensions Before and After Passage Through Ion Exchange Resin of Varying Supernatant pH.

20 % tissue suspension	pH of resin supernatant	pH of tissue suspension		Total N. mg/ml	
		Before	After	Before	After
Chick embryo	5.8	7.1	6.9	1.21	.08
	6.8	7.1	7.7	1.21	.13
	7.8	7.1	8.1	1.21	.23
	8.8	7.1	9.8	1.21	.20
,, ,,	5.8	7.2	6.9	1.23	.10
	6.8	7.2	7.7	1.23	.18
	7.8	7.2	8.0	1.23	.17
	8.8	7.2	9.9	1.23	.24
Mouse brain	5.8	7.2	7.1	2.22	.02
	6.8	7.2	7.7	2.22	.20
	7.8	7.2	7.9	2.22	.35
	8.8	7.2	9.9	2.22	.65
,, ,,	5.8	7.1	7.2	2.18	.09
	6.8	7.1	7.8	2.18	.28
	7.8	7.1	8.3	2.18	.35
	8.8	7.1	9.7	2.18	.77

5 mice for each serial 10 fold dilution performed in the usual manner with the results expressed as LD_{50}.

Results. In Table I are shown the nitrogen content and pH values of normal chick embyro and mouse brain tissue suspensions (20%) before and after passage through resin columns adjusted as described over a pH range from 5.8 to 8.8. It will be seen that in both pairs of experiments there was a reduction in the nitrogen of the percolates amounting to 65 to 99%. Greater reductions in nitrogen were obtained at the lower pH values. Nevertheless, in experiments with infected tissues, the pH was adjusted to 6.8 or higher in order to keep within the optimum pH stability zones with respect to infectivity of the viruses studied(2,3,4).

The results of experiments in which infected tissue suspensions were passed through resin columns are shown in Table II. Suspensions of Japanese encephalitis virus obtained from infected chick embryos showed a reduction of 71 to 88% in total nitrogen after one passage through resin columns, while the infective titer was not reduced significantly except in one experiment. Somewhat less purification was obtained in the case of suspensions of Eastern equine encephalomyelitis virus obtained from infected chick embryos. These showed a reduction in total nitrogen of 50 to 76% and a reduction in infectivity titer of 0.1 to 0.6 log. The fact that somewhat more nitrogen was removed from Japanese encephalitis virus suspensions than from suspensions of Eastern equine encephalomyelitis virus with little or no loss in virus infectivity may have been due to differences in size or physico-chemical characteristics of the viruses under the conditions of these experiments.

Similar results were obtained with infected mouse brain suspensions. In these experiments 63 to 93% of the original total nitrogen content was removed while the reduction in infectivity titer ranged from zero with Japanese encephalitis virus to 1.3 log in the case of Eastern equine encephalomyelitis virus.

It should be noted that the reduction in virus infectivity titer was not in all cases directly proportional to the reduction in total

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2. Duffy, C. E., and Stanley, W. M., *J. Exp. Med.*, 1945, v82, 385.

3. Taylor, A. R., Sharp, D. G., and Beard, J. W., *J. Inf. Dis.*, 1940, v67, 59.

4. Olitsky, P. K., and Casals, J., in *Viral and Rickettsial Infections of Man*, ed. T. M. Rivers, J. B. Lippincott Co., Philadelphia, 1948, chap. 8, p. 183.

TABLE II.
Infective Titer and Total Nitrogen Content of Virus Infected Tissue Suspensions Before and After Passage Through Ion Exchange Resin.

	pH of resin supernatant	Infective titer Log dilution Before	 After	Total nitrogen Mg/ml Before	 After	 % reduction
	From chick embryo					
Japanese encephalitis—20%	7.8	6.6	6.6	0.96	.17	82
” ” ”	7.8	7.3	6.5	1.15	.25	78
” ” ”	7.8	6.4	6.5	0.80	.10	88
” ” ”	7.8	6.6	6.8	1.17	.24	79
” ” ”	7.8	7.5	7.4	1.10	.22	80
” ” ”	8.8	6.6	6.5	1.17	.34	71
Eastern equine encephalomyelitis—20%	8.8	9.1	8.5	1.24	.38	69
” ” ” ”	7.6	9.0	8.6	1.17	.59	50
” ” ” ”	7.6	9.6	9.5	1.12	.49	56
” ” ” ”	7.6	8.6	8.5	1.28	.31	76
	From mouse brain					
Japanese encephalitis—10%	8.8	7.4	7.5	1.12	.15	87
Eastern equine encephalomyelitis—20%	7.6	9.2	7.9	2.22	.16	93
Western equine encephalomyelitis—10%	7.6	9.0	7.8	1.27	.47	63
Rabies—5%	7.0	6.8	5.6	0.65	.17	74
” 10%	6.8	7.2	6.6	1.26	.25	80

nitrogen content. These differences may have been a result of variations in the flow rate through the columns.

Summary. Ion exchange resin techniques offer a simple and effective method for the partial purification of the neurotropic viruses which have been studied.

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Immunological Studies of Embryo-propagated Hamster-adapted Newcastle Virus in Chickens. (17640)

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A California strain of Newcastle disease virus, No. 11,914, adapted to the Syrian hamster(1), has proved effective in the form of hamster brain suspension in immunizing chickens against the virulent unmodified virus of this California strain(2), and against virulent Connecticut and Colorado strains(3). These successful immunizations prompted further experiments to test the value of this hamster-adapted strain after one passage in 8-day-old embryonated eggs. Vaccine used for this experiment was prepared from the 73rd hamster passage. Fifteen 5-week-old Syrian hamsters (average weight 40 g) were injected intracerebrally with 0.05 cc of a 10%

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2. Reagan, R. L., Lillie, M. G., Poelma, L. J., and Brueckner, A. L., *Am. J. Vet. Res.*, 1948, v9, 220.

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TABLE I.
Summary of Results Obtained with Living Hamster-Egg Newcastle Virus.

Vaccination 7/12/49					Symptoms & deaths		Challenge		Results	
Group	No. of birds	Vaccine	Amt in cc	Site	Typical	Atypical	Birds	Date	Died	Survived
1	60	Hamster-egg	.25	Base of wattle	5	4	51	8/8/49	0	51
1 RC	15	—	—	—	1	0	14	”	11	3
2	60	Hamster-egg	.01	Web of wing	2	0	58	”	2	56
2 RC	15	—	—	—	0	0	15	”	9	6

RC = Room control unvaccinated contact chickens.

brain suspension from infected hamsters of the above passage. Hamsters were sacrificed when typical symptoms of central nervous system involvement appeared in 48 to 72 hours. The brains of these hamsters were removed aseptically, ground with alundum, and diluted to a 10% suspension with physiological saline. Fifteen-hundredths cc of this suspension was injected into the allantoic sac of each of 124 8-day-old embryonated White Leghorn eggs. All eggs were incubated at 37.5° C. Fourteen eggs, dead after 24 hours incubation, were discarded. The allantoic and amniotic fluid were harvested and pooled from eggs that were dead in 48 to 96 hours. These eggs represented 69.09% of those alive after 24 hours. Later experiments showed that an increased volume of inoculum would cause a higher percentage of deaths at the proper interval. This harvested material titered $10^{-4.66}$ in eggs by the Reed and Muench calculation(4) and was used as the vaccine.

Healthy 6-week-old New Hampshire Red chickens, identified by wing bands, were used in the experiment. Hemagglutination-inhibition tests when performed with serum collected before vaccination were negative. Sixty chickens were injected in the base of the wattle with 0.25 cc of a 10% dilution of the virus-bearing allantoic fluid in physiological saline. Fifteen unvaccinated chickens were placed with the vaccinated birds as room contact controls. These 75 birds comprised Group I.

A second series of 60 chickens was vaccinated by the stick or wing-web method with concentrated allantoic fluid. Fifteen unvaccinated chickens were placed with these vaccinated birds as room contact controls. These 75 birds were designated as Group II. Careful observations of both groups were made daily. Results of the 27-day observation period before challenging are shown in Table I.

After 27 days birds of both groups were challenged in the base of the wattle with 0.5 cc of a 10^{-1} dilution of allantoic and amniotic fluid from a virulent California strain (No. 11,914) of Newcastle disease virus, which had been carried through 43 passages in embryonated chicken eggs. Normal susceptible birds of corresponding age were challenged at the same site of inoculation with the same material. The results obtained after challenge are shown in Table I. The challenge material titered 10^{-5} in 7-day-old embryonated chicken eggs and $10^{-4.47}$ in 6-week-old chickens as calculated by the method of Reed and Muench(4).

Discussion. The allantoic fluid from the hamster-egg passage, used as a vaccine, was pathogenic for chickens to the extent of 8.33% by subcutaneous inoculation, and 3.33% by the stick method. Of the normal room controls added to the vaccinated chickens before challenging, 3.33% contracted Newcastle disease from vaccine contact; and 68.96% of those remaining became infected when challenged with Newcastle disease virus. On challenge, no chickens of the group vaccinated subcutaneously showed evidence of Newcastle disease, while 3.44% of chickens in the stick method vaccinated group became infected.

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4. Reed, L. J., and Muench, H., *Am. J. Hyg.*, 1938, v27, 493.

Summary. The immunizing value of a living vaccine prepared from the first hamster-egg passage was of a high order against challenge by injection. Although a slightly lower degree of protection was obtained, the vaccine is considerably less pathogenic when given by the stick or wing-web method of vaccination.

Acknowledgment for technical assistance is given to Mr. William Blum and Miss Helen L. Reagan.

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Eosinopenia Due to Glucose Administration.* (17641)

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In recent years the physiology of the adrenal cortex has received enormous consideration because of its importance in maintaining homeostatic equilibrium under normal conditions as well as during periods of stress. It is generally considered that the activity of the adrenal cortex is under the influence of adrenocorticotrophic hormone which is liberated by the anterior pituitary. Forsham *et al.*(1,2) believe that the depression of the circulating eosinophiles of the blood is a sensitive physiological criterion of increased adrenal cortical activity. They have shown that 11-oxygenated corticosteroids, ACTH, and epinephrine(3) will decrease the eosinophile count in subjects with normally active adrenal cortical tissue, but that the response is absent when this tissue is functionally deficient. The eosinopenia, which is maximum about 4 hours after adrenal cortical stimulation, is accompanied by an increased renal excretion of sodium, chloride, potassium, uric acid, 17-ketosteroids, and 11-oxysteroids, which is further indication of increased adrenal cortical activity. Pincus *et al.*(4) have shown that oral administration of glucose in the rat produced a lymphopenia which was synchronous with the rise in the blood sugar. This was interpreted as evidence of increased adrenal cortical discharge. A significant correlation was noted between the magnitude of the hyperglycemia and the lymphopenic response. Jailer(5), using comparable doses of glucose in normal man, found that there was no such correlation and that the changes in the lymphocyte levels were within the limits of experimental error. Forsham *et al.*(1) have also cast doubt upon the validity of lymphopenia as an index of adrenal cortical discharge, since they demonstrated an overlap in this response in Addisonian and non-Addisonian patients following ACTH administration. The recent observations of Steeples(6) that adrenal cholesterol increased within 30 minutes following the oral administration of glucose in the rat, was interpreted as indicative of adrenal cortical inhibition. The variance in these results motivated us to evaluate the action of hyperglycemia in terms

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[*] These studies were supported by grants from the Office of the Surgeon General, Department of the Army, and the Chicago Heart Association.

[†] Public Health Service Research Fellow of the National Heart Institute.

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2. Thorn, G. W., Forsham, P. H., Prunty, F. T. G., and Hills, A. G., *J. A. M. A.*, 1948, v137, 1005.

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5. Jailer, J. W., Marks, D. T., and Marks, P. A., *J. Clin. Endocrinol.*, 1948, v8, 1074.

6. Steeples, G. W., and Jensen, G., *Am. J. Physiol.*, 1949, v157, 418.

TABLE I.
Variability of the Eosinophile Count in Normal Dogs.

	Eosinophil counts (cells/mm^3)						
		Hr after tapwater stomach tubing					
Dog	Control	1	2	3	4	5	Adjusted c.v.,* %
B	1078	1078	1133	1122	1056	1034	± 6.2
Q	2266	2431	2068	2178	2244	2354	± 11.4
C	1298	1298	1474	1188	1155	1144	± 20.0
H	1771	1870	1738	1815	1859	—	± 6.6
G	1991	2167	2233	2233	2244	—	± 9.6
Qi	2079	2090	1683	1837	2145	1958	± 15.2
S	1177	1133	1188	1177	1133	1056	± 8.0
T	2178	2332	2475	2343	2387	2101	± 12.0

* Coefficient of variation.
Mean of 8 adjusted c.v. 11.1%.

of another index of increased adrenal cortical activity, namely the circulating blood eosinophiles.

Methods: Hyperglycemia was produced in two ways. Glucose was administered orally to trained unanesthetized dogs by stomach tube in doses of 2, 4, and 8 g per kilo body weight. Each dose was dissolved in 200 cc of water and given in 2 portions 30 minutes apart. Hyperglycemia was also produced by the intravenous infusion of a 15% glucose solution at the rate of 8 cc/min. for periods up to 6 hours. Plasma glucose determinations were made by the method of Nelson(7) using a Somogyi filtrate.(8) Eosinophile counts were made using a modification of the acetone-eosine method of Dunger.(9) Four Spencer Bright line chambers were counted in each determination. The maximum variability allowed between chambers in control counts was 10%. The average of four chambers was multiplied by a factor of 11.1 to give the number of cells/mm^3 of blood. In order to evaluate the variability of our techniques, control counts were done on 8 dogs before and after stomach tubing with tap water. Counts were then repeated at hourly intervals on each dog for 5 hours. A coefficient of variation (c.v.) for the 6 counts in each dog was calculated. Each c.v. was multiplied by 2 to yield an adjusted c.v. so that the average of the 6 counts ± this value would include 95% of a given dog's counts. The mean of the eight adjusted coefficients of variation was found to be ± 11.1%.

Results. The control stomach tubing procedure did not appreciably alter the number of circulating eosinophiles in this series of trained dogs (Table I). These data were of value in estimating the variability of the eosinophile count as observed in our laboratory. Since the absolute eosinophile counts varied markedly from dog to dog, eosinopenic reactions to stress were made in terms of per cent deviations from an average control count for each dog. Alterations in the circulating blood eosinophiles and changes in plasma glucose levels in six unanesthetized dogs following glucose tolerance tests at three dosage levels are presented in Fig. 1. These data indicate that oral glucose in doses of 2, 4, and 8 g/kg will produce a significant eosinopenia occurring 2.5 to 4.5 hours after the administration of glucose. A given dose of glucose did not evoke a comparable eosinopenic response in every dog, however progressive increases in hyperglycemia with increasing doses of glucose produced progressive decreases in the eosinophile counts. Analysis of the 4 hourly eosinopenic responses following glucose administration revealed statistically significant differences between both the 2 and 8 g doses and the 4 and 8 g doses. The differences in eosinopenia effected by 2 and 4 g of sugar were not statistically valid. The eosinophile responses to the 3 levels of oral glucose

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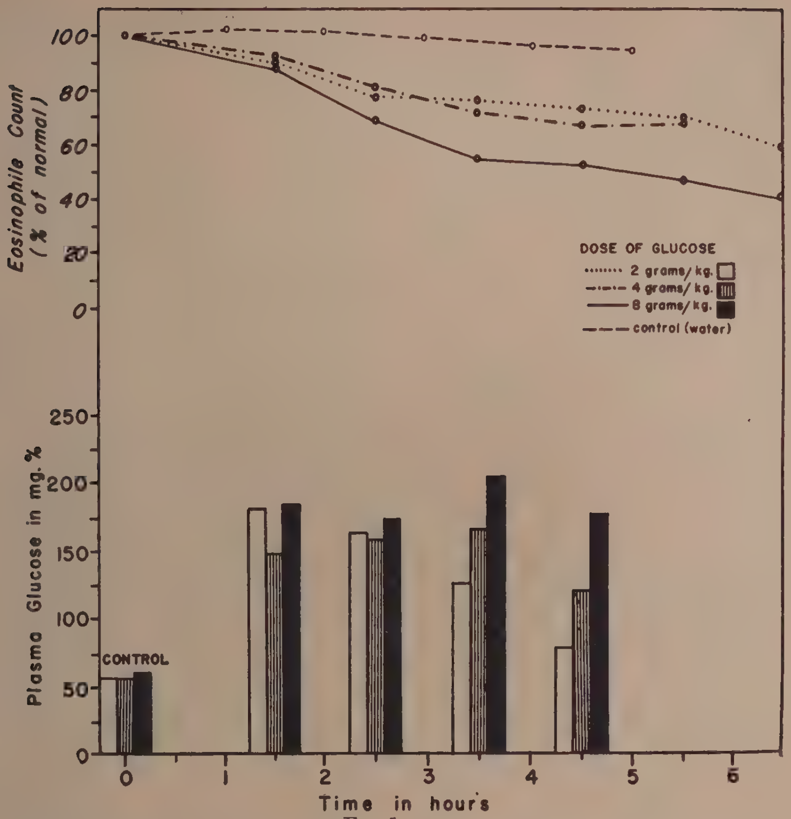

FIG. 1.
Mean eosinophile responses following the oral administration of glucose in 6 dogs.

yielded a parabolic type of dose-response curve.

The mean eosinophile and plasma glucose changes in 7 dogs following the infusion of 15% glucose at 8 cc/min. for 6 hours are illustrated in Fig. 2. The plasma glucose concentration reached a plateau after the first two hours of infusion, while the eosinophile counts continued to fall throughout the infusion. The constancy of hematocrit values after the first hour of glucose infusion obviated the possibility that the decrease in circulating eosinophiles was due to progressive hemodilution.

Discussion. There is evidence to the effect that exposure to stress results in the release of adrenocorticotrophic hormone from the anterior pituitary which in turn stimulates the secretion of the adrenal cortex. That the eosinopenic response is a manifestation of this reaction is indicated by its failure to appear in animals with adrenal cortical insufficiency. The data presented here suggest that hyperglycemia leads to an activation of the adrenals. The hyperglycemia response to oral glucose rose to a maximum within the first 1½ hours and gradually subsided over a 5 hour period. The eosinophile response, however, was maximum after 2.5 to 4.5 hours. Temporally speaking, the hyperglycemic and eosinopenic reactions in the dog following the oral administration of glucose were asynchronous. This is in contrast to the work of Pincus in the rat and man where the height of the blood sugar response coincided with the maximum lymphopenic reaction at the end of one half hour. The maximum eosinopenic response reported here, occurring at about 4 hours, is in

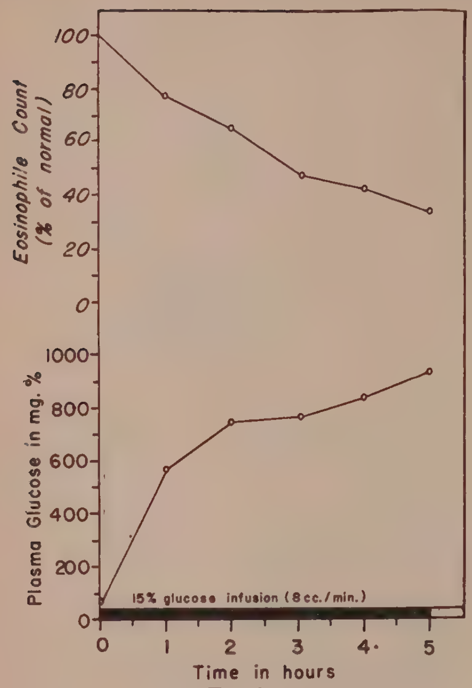

FIG. 2.

Mean eosinopenic responses in dogs during intravenous infusion of 15% glucose solution.

keeping with the response following the administration of epinephrine, ACTH, or C_{11} steroids.

These data do not elucidate the mechanism of action of hyperglycemia in effecting adrenal cortical discharge. It is conceivable as suggested by Vogt(10) and Goldlowski(11) that hyperglycemia may initiate the release of insulin which may directly stimulate the release of adrenocorticotrophic hormone from the anterior pituitary.

Summary. 1. The absolute eosinophile count in the dog varies markedly from animal to animal. The variability of the eosinophile count in the dog is reflected by a mean adjusted coefficient of variation of ± 11.1%.

2. Hyperglycemia produced by the oral or intravenous administration of glucose in the unanesthetized dog causes an eosinopenic response presumably mediated through activation of the pituitary-adrenal mechanism. In this respect hyperglycemia may be construed as a non-specific stress factor.

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Susceptibility of Newborn Mice of an Otherwise Apparently "Resistant" Strain to Inoculation with Leukemia.* (17642)

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A strain of transplantable lymphatic leukemia that originated spontaneously(1) in an 11-month-old female of our colony of Ak mice† has been maintained in our laboratory by successive intraperitoneal inoculations of Ak mice; this strain, at the present time in its 52nd transfer, has been designated "Ak Leukemia." Small pieces of liver, spleen, and lymph glands, removed aseptically from Ak mice that had developed leukemia as a result of inoculation, were weighed, then cut and ground in a sterile mortar, 0.85% solution of sodium chloride being added to obtain cell suspensions of 20% concentration. Only such cell suspensions were used for inoculations in experiments reported in this study; the potency of these cell suspensions, at least for mice of the Ak line, was such, however, that dilutions exceeding 1:10,000 reproduced regularly acute leukemia when inoculated into the peritoneum, or under the skin of mice, of either sex and any age, of the Ak line. The pathogenic potency of cell suspensions of Ak leukemia appeared, at first, to be limited only to mice of the Ak line. In this respect this strain of leukemia did not seem to differ from many other tumors that develop in mice of pure inbred lines. A few months ago, however, as a part of an experimental project(2) only indirectly related to the present study, a group of mice of a C3H subline‡, consisting of both adult parents and their 5 infant mice 24 hours of age, was inoculated subcutaneously with Ak leukemia: the infants received 0.05 cc, the parents 0.25 cc, each, of the leukemic cell suspension. As a result, all 5 infants developed acute leukemia within 12 days after the inoculation; their parents, however, remained in perfect health. This accidental observation prompted a systematic study, the results of which can be summarized as follows:

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* Reviewed in the Veterans Administration and published with the approval of the Chief Medical Director. The statements and conclusions published by the author are the result of his own study and do not necessarily reflect the opinion or policy of the Veterans Administration.

1. Cole, R. K., and Furth, J., *Cancer Research*, 1941, v1, 957.

† Our colony of Ak mice has been raised from a litter of Ak-n mice obtained in 1945 from Dr. J. Furth, Cornell University, New York City.

Eighty-five suckling mice, 1 to 7 days old, of either the C3H line§ or of the An (C3H) subline‡ were inoculated subcutaneously (0.05 cc each) with Ak leukemic cell suspensions; in 82 of these 85 baby mice, subcutaneous tumors developed within 12 days at the site of inoculation, followed by a generalized and fatal leukemia. This striking susceptibility of the suckling infant mice to inoculation with leukemia, was found, however, to diminish rapidly after the seventh day of life. Thus, when 57 suckling mice, 8 to 15 days old, of either the C3H or An line, were inoculated subcutaneously (0.05 to 0.2 cc) with AK leukemia, only 27 of them developed tumors at the site of inoculation; three of these tumors regressed spontaneously; the others were followed by a generalized leukemia.

Leukemia resulted less frequently when older infants were used for the tests: 60 infant mice, 16 to 30 days old, of the same inbred lines, were injected subcutaneously or intraperitoneally with doses varying from 0.05 to 0.3 cc of Ak leukemia; only in 15 mice local tumors resulted from these inoculations; of these, 5 regressed spontaneously; the others, however, progressed into a generalized leukemia.

Adult mice, 2 to 6 months old, of both the C3H and An lines, were found to resist either subcutaneous or intraperitoneal inoculations of substantial doses of Ak leukemia. Forty-nine mice were inoculated subcutaneously, and 9 intraperitoneally, with doses varying from 0.2 cc to 1 cc of the leukemic cell suspensions; none of the 58 inoculated animals developed local tumors or leukemia.

Further experiments demonstrated, however, that the resistance of the adult mice of either the C3H or An line to inoculation with Ak leukemia could be broken, provided that overwhelming doses were injected, in particular by the intraperitoneal route of inoculation. Thus, 5 adult (2 month old) females of the C3H line were inoculated with massive doses subcutaneously, each mouse receiving 3 cc of the leukemic cell suspension. In 3 animals small subcutaneous tumors developed within 10 days at the site of inoculation, but 2 of them regressed spontaneously; only 1 of these progressed eventually into a generalized leukemia. When, however, 6 adult (2 month old) females of the C3H line were inoculated intraperitoneally with similar, massive doses (3 cc each) of the leukemic cell suspensions, all 6 animals died from leukemia within 12 days following the inoculation.

Those mice of either the C3H line, or of the An (C3H) subline that did not apparently react to the inoculation of Ak leukemia, have been under observation for periods of time not yet exceeding 4 months; they now appear to be in perfect health. It remains to be seen, nevertheless, whether all of them will remain free from leukemia for the balance of their lives.

Summary. 1. A strain of transplantable lymphatic leukemia which originated in a mouse of the Ak line was found to be highly

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‡ A colony of foster nursed mice of a C3H subline, free from the mammary carcinoma agent, has been raised in our laboratory (and designated by the symbol "An") from a litter obtained in 1945 from Dr. H. B. Andervont, National Cancer Institute, Bethesda, Md.(3)

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3. Gross, L., *J. Immunol.*, 1947, v55, 297.

§ Our colony of C3H mice has been raised from a litter obtained in 1944 from Dr. J. J. Bittner, University of Minnesota.(3)

pathogenic for suckling infants of the C3H inbred line. Cell suspensions (20%) prepared from liver, spleen and lymph glands of leukemic mice of the Ak line, reproduced acute leukemia within 12 days when inoculated under the skin (0.05 cc) of newly born suckling mice of the C3H line.

2. This striking susceptibility of suckling C3H infants to inoculation with Ak leukemia appears to be limited to their first few days of life. Practically all infants of the C3H line (82 out of 85) developed leukemia when inoculated within the first 7 days of life; only 27 out of 57, however, were found to be susceptible when inoculated at ages varying from 8 to 15 days. Of 60 infants, 16 to 30 days old, only 15 reacted to inoculation.

3. Forty-nine adult mice of the C3H line were inoculated subcutaneously and 9 intraperitoneally with large doses (0.2 to 1 cc) of Ak leukemia and none reacted. When, however, overwhelming doses (3 cc each) of the Ak leukemic cell suspension were injected into adult mice of the C3H line, 1 out of 5 inoculated subcutaneously, and all 6 inoculated intraperitoneally died from leukemia.

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Inhibition of Hyaluronidase *in vivo* by Adrenal Cortical Activation. (17643)

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Among the actions of the adrenal steroids is the inhibition of the spreading effect of hyaluronidase injected into the skin(1). We have attempted to measure the decrease in hyaluronidase activity following stimulation of the adrenal cortex in the human. Of the various methods employed to measure hyaluronidase activity, the skin diffusion technic seemed the most practical for wide clinical application. In this study, a dilute preparation of fluorescein was used as an indicator dye for intracutaneous injection with the enzyme. Fluorescein was shown by Bukantz and Dammin(2) to be satisfactory for evaluating the effect of antihistaminic drugs upon the action of histamine. Activation of the adrenal cortex following the administration of epinephrine was established by Vogt(3) and Long(4); the possible magnitude of these responses being studied also by Vogt. The mode of stimulation of the adrenal cortex through the anterior pituitary(4) and, perhaps hypothalamus(5) has been shown. In the present study, epinephrine has been used to stimulate the pituitary-adrenal system in control patients in order to demonstrate inhibition of hyaluronidase injected intracutaneously when the adrenal cortex is activated.

Method. A test for skin diffusion was performed using fluorescein (1:25,000) and hyaluronidase* (150 TRU/cc) drawn into a tuberculin syringe for intracutaneous injection. From 0.01 to 0.03 cc of the enzyme are added to the dye to produce a volume of 0.1 cc which is injected into the volar surface of the forearm. The endpoint for the test is noted when the presence of the dye in the skin cannot be appreciated. It is essential to have either daylight or ultraviolet light in the form of a G.E. Purple X bulb or a Wood's lamp to observe the dye disappearance. Gentle stroking with an alcohol sponge enhances the color and the wheal produced by the injection. The test has been done in the fasting state before and 4 hours after a subcutaneous injection of 0.3 cc of 1:1000 epinephrine. Other dyes have been used in an effort to improve on

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1. Opsahl, J. C., *Yale J. Biol. and Med.*, 1949, v21, 255.

2. Bukantz, S. C., and Dammin, G. J., *Science*, 1948, v107, 224.

3. Vogt, M., *J. Physiol.*, 1944, v103, 317.

4. Long, C. N. H., *Fed. Proc.*, 1947, v6, 461.

5. Hume, D. M., *J. Clin. Invest.*, 1949, v28, 797.

* Wyeth, Inc., Hydase, supplied by Dr. George E. Farrar.

the endpoint obtained with fluorescein but none have yet been found. The results obtained with fluorescein can be duplicated by the same observer but experience with it is necessary before the test can be evaluated easily. The use of dermofluorometer or skin microscope may resolve this difficulty.

The epinephrine test described by Forsham, *et al.*(6) was performed on 20 patients who were free of acute or debilitating diseases. In addition, there were 3 cases of suspected hypoadrenocorticism tested, and a number of other patients with chronic diseases, or with acute processes which would constitute a background for adrenocortical activity prior to the test. Several observations were made using an aqueous solution of adrenal extract (Upjohn) intra-arterially to note the effect of a high concentration of steroid in the area supplied by the artery.

Results. The time required for fluorescein to disappear from the skin when injected alone averaged 40 minutes. When hyaluronidase is added to fluorescein the color disappears in 4 minutes with a range from 3 to 7 minutes. In 20 patients tested with fluorescein and hyaluronidase, 4 hours after epinephrine the average disappearance time of the dye had increased from 4 minutes to 21 minutes with a range of 15 to 31 minutes. The results of the fluorescein-hyaluronidase skin tests were found to agree with eosinophile response in this group. The average fall in the total eosinophile count was 68.2%. However there were several instances where the fall in eosinophiles was less than 50%. It will be noted that a significant degree of inhibition of the diffusing effect of hyaluronidase was achieved during a period of epinephrine-induced adrenocortical activity as gauged by the eosinophile response. In three control patients hyaluronidase inhibition occurred without the expected eosinophile response. Previous experience(7) has shown that patients with therapeutic blood levels of salicylates or heparin will inhibit the spreading effect of hyaluronidase. There is reason to suspect that digitalis, tocopherols, and ascorbic acid will do the same. One patient receiving salicylate therapy in the present study also showed inhibition of the enzyme initially to 14 minutes which was doubled following epinephrine. This patient had a 90% fall in eosinophiles at the same time. Ascorbic acid with desoxycorticosterone acetate in four cases slightly inhibited hyaluronidase but did not affect the eosinophile count. Intraarterial adrenal steroid was found to inhibit hyaluronidase in the area supplied by the artery.

Following surgery or other procedures of a stressful nature there was inhibition of the hyaluronidase action similar to or greater than that obtained with epinephrine. This finding was associated with a low eosinophile count and indicated a normal pituitary-adrenal response. In three patients with hypoadrenocorticism with low 17-ketosteroid excretion there was no fall in eosinophiles using the epinephrine test. Nor was there any inhibition of hyaluronidase in these patients as measured by the disappearance of fluorescein injected with it. (Table I)

Discussion. Inhibition of hyaluronidase by the administration of adrenal steroids or by stimulation of the adrenal cortex has been demonstrated by several workers. Opsahl(1) found rapid spread of India ink in adrenalectomized animals and that ACE reduced the spread in these animals. An increase in serum inhibitors of hyaluronidase has been noted by Glick(8) following the administration of ACTH. Seifter *et al.*(9) have found that certain steroids or the serum of animals given alarming stimuli will inhibit hyaluronidase *in vitro* and *in vivo*. The inhibition of the spread of dyes by ACE was originally observed by Menkin(10). By use of the technic described in this paper it has been possible to test the functional response of the adrenal cortex to epinephrine or other stimuli in patients. Stimulation of the adrenal cortex results in inhibition of hyaluronidase activity as measured by

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7. Shuman, C. R., in press.

8. McQuarrie, I., Bank, E. G., Ziegler, M. R., and Wright, W. S., PROC. SOC. EXP. BIOL. AND MED., 1949, v71, 555.

9. Seifter, J., Balder, D. H., and Dervinis, A., PROC. SOC. EXP. BIOL. AND MED., 1949, v72, 136.

10. Menkin, V., *Am. J. Physiol.*, 1940, v129, 691.

TABLE I.
Factors Influencing the Disappearance Time of Fluorescein and Hyaluronidase.

cc	Injected	No. of patients	Disappearance time of dye		Eosinophile fall (avg %)
			Avg (min.)	Range	
0.10	F*	26	40	36-64	
0.09	F + H† 0.01 cc	42	4	3-7	
0.09	F and H 0.01 cc 4 hr after 0.3 cc epinephrine				
	A. Control	20	21.7	16-31	68.2 (3 <50%)
	B. Hypoadrenocorticism	3	4.7	1-8	17.5
0.09	F and H 0.01 cc 20 min. after 1 g ascorbic acid I.V. + DCA I.M.	4	20	15-30	
0.09	F and H 0.01 cc 4 hr after operation	2	25	20-30	90
0.09	F and H 0.01 following intra-arterial adrenal cortical extr.	3	12	10-14	

* F = Fluorescein.
† H = Hyaluronidase.

the disappearance of the indicator dye. The use of this test as a method for screening the integrity of the pituitary-adrenal system is suggested. Since none of the initial disappearance times with hyaluronidase were over 8 minutes it is probable that if the time of dye disappearance after epinephrine is longer than 12 minutes the adrenal cortex is functioning normally. Tentatively, on the basis of our observations in 3 patients, it is suggested that if the dye disappears in less than 12 minutes when injected 4 hours after epinephrine, the adrenal cortex is unrepsonsive. None of the drugs known to inhibit hyaluronidase should be given at the time of or prior to the test.

One factor which needs to be evaluated in the response to intracutaneous hyaluronidase is the age of the patient. Our experience has shown that elderly patients show slower diffusion with hyaluronidase than do the younger patients. Perhaps the substrate, hyaluronic acid, in the skin of older patients is more resistant to hydrolysis than that of younger patients. The state of hydration of the control patients was relatively good. It will be necessary to observe the effect of dehydration and edema upon the disappearance time of the indicator dye in order to evaluate the influence of these factors upon the proposed test. The effect of feeding upon the results of the skin test has not been studied. This may prove to be an advantage over the eosinophile count since there is no reason to anticipate that feeding will in any way inhibit the diffusing action of the enzyme. The influence of a variety of steroids upon hyaluronidase action is being studied by the technic described. The present opinion is that certain steroid hormones inhibit the spreading effect of hyaluronidase by producing a change in the hyaluronate substrate of the tissues. It has not yet been possible to demonstrate how the steroids affect ground substance to block the action of the enzyme. The possibility of direct inhibition of the enzyme by steroid hormones or by serum inhibitor activated by these hormones must be considered. The relationship of the interactions between the steroid hormones and the hyaluronate-hyaluronidase system to the collagen diseases may be a fruitful field for investigation.

Summary. The inhibition of hyaluronidase injected into the skin of patients with normal pituitary-adrenal responses to epinephrine has been observed. The diffusing effect of hyal-

uronidase was gauged by the disappearance of fluorescein dye injected with it intracutaneously. The failure of antagonism to the action of hyaluronidase to occur in patients with hypoadrenocorticism was noted. The possibility of utilizing the method described as a test for pituitary-adrenal response to epinephrine is discussed.

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Effects of Reichstein's Compound S, Pregnenetriolone Acetate, and 17-Alpha Hydroxyprogesterone in Rheumatoid Arthritis. (17644)

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The promising results of Hench, Kendall, and Polley(1) with 17-hydroxy 11-dehydrocorticosterone (Compound E) in patients with rheumatoid arthritis and more recent ample confirmation(2,3) suggested that other related steroid compounds might also be of value in this disease. The steroids used in this study, 17-Alpha Hydroxy-11-Desoxycorticosterone-21-Acetate (Reichstein's Compound S Acetate), 17-Hydroxyprogesterone (17-Alpha Hydroxyprogesterone), and 4-Pregnene-17-, 20,21-Triol-3-One-20-21-Diacetate (Pregnenetriolone Diacetate) were made available to us by Dr. James A. Shannon, of the National Heart Institute, who also supplied us with their formulation. The compounds were dried and screened and suspended* in a sterile aqueous menstrum containing .25% Tween 80.

Two patients with classical rheumatoid arthritis were treated with each of the above compounds in the dosage of 100 mg daily for 7½ days (750 mg), and 400 mg daily for 4½ days (1750), divided into 6 daily doses and given at 4 hour intervals intramuscularly. All patients received placebo therapy for at least one week before steroid therapy was begun. Metabolic balance studies were not carried out but each patient was placed on a constant diet at least one week before control studies were begun. Serum chlorides, potassium, sodium, carbon dioxide combining power, urea nitrogen, uric acid, and fasting blood sugar determinations were estimated twice during the control period and after each period of therapy. The twenty-four hour urinary excretion of sodium, potassium, urea, chloride, creatinine, and uric acid was estimated in the same fashion. Intravenous glucose tolerance tests were done on all patients during the control period and after therapy. Hemoglobin, hematocrit, sedimentation rate, white blood count, differential, and total eosinophil counts were done twice on all patients during the control period and after each study period.

In addition to the laboratory data, each patient was very carefully followed by the same physician with daily weight, blood pressure, and estimation of degree of swelling and limitation of motion of each involved joint.

Results. In no case was objective improvement of any involved joint observed. Subjective improvement was fleeting and all patients felt worse by the end of the study. In one of the patients treated with Compound S Acetate definite swelling, heat, and tenderness appeared in a previously uninvolved joint during therapy. There was no change in the blood pressure or weight of any of the patients. There were no changes in any of the hematologic or chemical data observed during the course of therapy.

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* Suspensions made through the courtesy of Hynson, Westcott & Dunning.

Summary. The 3 steroid compounds, Pregnenetriolone Acetate, 17-Alpha Hydroxyprogesterone, and Compound S of Reichstein, in the doses used in this study, had no effect on any of the observed clinical, chemical, or hematological manifestations of six classical cases of rheumatoid arthritis.

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Action of Chloromycetin and of Aureomycin on Normal Tissue Cultures.

(17645)

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The two new antibiotics, chloramphenicol (Chloromycetin) and aureomycin, which have been shown to be efficacious in the treatment of a number of infectious diseases(1,2), are perhaps of especial interest because of their capacity to inhibit multiplication of rickettsiae and certain viruses. This activity against agents which maintain an obligate intracellular parasitic existence led us to investigate the effect of the antibiotics on the proliferation of normal cells in tissue cultures.

Materials and methods. Tissue culture method. Chick embryo tissues were cultivated on a plastic membrane in a liquid medium by a technique described elsewhere(3,4). Fifteen-day-old embryos were used when the explants were prepared from heart or lung and 11-day embryos when mesencephalon provided the tissue. The liquid medium, containing defibrinated plasma, embryo extract, and Tyrode's solution, was changed on the third day of incubation. Under these conditions of cultivation, the proliferating cells grew in a flat single layer sheet and consisted almost entirely of fibroblasts from the cardiac tissue and of epithelial cells from the lung tissue. The amount of growth of tissue was estimated on the fifth day in the following manner: Using a camera lucida, the margins of the original explant and of the proliferated tissue were outlined on paper of uniform thickness. The pattern of the new growth exclusive of the explant was cut out with scissors. These patterns from the 5 to 16 duplicate cultures in each individual test were weighed and the average value taken as the coefficient of new growth. Explants from the same batch of embryos were used in a given experiment which consisted of control cultures and test cultures containing the designated amounts of antibiotic. The coefficients of growth for the control cultures differed in each experiment despite the care exercised in selecting explants of approximately the same size and shape and in eliminating other variables. Nevertheless, the trend of results obtained in an experiment with varying amounts of antibiotic were reproducible.

Antibiotics. A crystalline preparation of chloramphenicol (Chloromycetin, Lot X 3143), provided by Parke, Davis and Company, and a preparation of aureomycin hydrochloride (Duomycin, Lot 7-8411), provided by Lederle Laboratories, were used in the present work. The drugs, dissolved in physiological saline solution, were added to the liquid media used in the tissue cultures to give the final concentrations desired. All media used in a test, including that for the control cultures, was diluted to the same proportion with saline solution. The presence of chloramphenicol even in concentrations of 1,000 γ/ml had no effect on the pH of the media and caused no precipitate. On the other hand, a slight turbidity was noted in media containing 100 γ/ml of aureomycin and a precipitate in that containing 1,000 γ/ml. Further-

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more, the presence of these concentrations of aureomycin caused the pH of the media to drop from 7.3-7.4 to slightly below 7.0 but the pH returned to 7.2 when the mixtures were incubated overnight. The bacteriostatic effect of the two antibiotics was tested against the Pasteur Institute strain of *Escherichia coli* under various conditions. The minimal concentration of chloramphenicol which caused complete inhibition of growth of the organism for 24 hours was between 2.5 and 5.0 γ/ml irrespective of whether the substrate was Ringer's solution, Tyrode's solution or the special liquid medium used for tissue cultures. Furthermore, in the tissue culture media the values were the same when the mixture was tested fresh or after storage at 4° or 37°C for 24 hours. Under similar conditions the inhibitory level of aureomycin was 10.0 to 50.0 γ/ml. These findings are in agreement with those of Smith and his coworkers(5) with chloramphenicol but the inhibitory range of aureomycin for the present organism differs somewhat from the observations of Bliss and Chandler(6) with aureomycin on their strain of *E. coli*. The two sets of observations do not agree on the effect of proteins in the media and of temperature on the deterioration of aureomycin. The Baltimore workers who used a different method concluded that the antibacterial effect of this antibiotic was greatly diminished in the presence of serum and by storage at 24° or 37° C. The fluid of the test tissue cultures was replaced on the third day with liquid media containing fresh antibiotic.

Results. Data on the inhibitory effect of various concentrations of chloramphenicol and aureomycin on the growth of fibroblasts and epithelial cells *in vitro* are summarized in Table I. There was a slight but consistent diminution in proliferation of both types of cells in the presence of 10 γ/ml of chloramphenicol. The effect was marked when the concentration of this drug was 100 γ/ml and essentially no growth was noted in the cultures containing 1,000 γ/ml. Microscopic examination of the tissue cells revealed no change in appearance in those grown in 10 γ/ml. However, the cells from the cultures containing 100 γ/ml showed numerous vacuoles and fatty degeneration. Moreover, the proliferating cells no longer grew in a smooth flat sheet. The fibroblasts formed a loose network and the epithelial cells tended to pile up at the thickened peripheral margin. The few fibroblasts and epithelial cells which succeeded in proliferating in the cultures containing 1,000 γ/ml underwent degeneration terminating in death.

The effect of aureomycin on the growth of epithelial cells is of the same order as that of chloramphenicol, see Table I. The data suggest that fibroblasts are more readily inhibited by aureomycin than are epithelial cells.

Similar experiments were made with tissue cultures seeded with tissue from the mesencephalon. The results of these tests, which are summarized in Table II, are in agreement with those in which heart and lung were employed in that concentrations of 100 γ/ml of either antibiotic markedly inhibit cell migration and 1,000 γ/ml prevents it. In this experiment 10 γ/ml of chloramphenicol had an effect whereas the same concentration of aureomycin did not.

Discussion. Concentrations of chloramphenicol which appreciably inhibit *in vitro* proliferation of fibroblasts, epithelial cells and cells from explants of embryonal brain tissue are within the range attained in the blood of patients who receive therapeutic amounts of this antibiotic(7). While less voluminous data are available on the blood levels of aureomycin in patients being treated with this drug(8), the levels approach those which inhibit growth of tissue cells *in vitro*. It may well be that the present observations are not applicable to mature cells or to proliferating

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TABLE I.
Effect of Chloramphenicol and Aureomycin on Growth of Tissue Cultures.

Chick embryo tissue	Type of cell growth	Antibiotic		Experiment 1		Experiment 2	
		Type	Concentration, γ/ml	Coef. of new growth	No. of cultures	Coef. of new growth	No. of cultures
Heart	Fibroblast	Control	0	20	8	36	12
		Chloramphenicol	10	17	7	28	15
		"	100	8	14	9	15
		"	1000	0.5	14	0	8
		Control	0	63	14		
		Aureomycin	1	53	10		
		"	10	48	11		
		"	100	2	5		
		"	1000	0	10		
Lung	Epithelial	Control	0	17	12	19	15
		Chloramphenicol	10	14	8	11	13
		"	100	10	15	4	16
		"	1000	3	14	—	—
		Control	0	34	11		
		Aureomycin	1	32	15		
		"	10	29	12		
		"	100	22	7		
		"	1000	0	12		

TABLE II.
Effect of Antibiotics on Proliferation of Cells from Explants of Brain.

Antibiotic	Conc., γ/ml	Coef. of new growth	No. of cultures
Control	0	20	12
Chloramphenicol	10	13	12
"	100	12	12
"	1000	0	10
Aureomycin	10	19	11
"	100	8	12
"	1000	0	10

cells in the living host. However, the phenomena are worth considering particularly in patients who require repair of tissue and who are maintained on prolonged therapeutic regimens.

The observations that concentrations of 100 γ/ml of chloramphenicol are directly toxic for cells growing in tissue culture, as shown by histological changes, are in accord with the finding that the LD_{50} of chloramphenicol in 6-day embryonated eggs is approximately 1.5-2.0 mg(9). In the latter instance the over-all concentration of drug in fluids and tissues of the embryo is of the order of 30-40 γ/ml. It may be pointed out that patients with blood levels of 100 γ/ml of chloramphenicol for short periods of time have not shown toxic manifestations(7).

Summary. Proliferating epithelial cells and fibroblasts, and cells from explants of embryonal brain tissue are retarded in growth *in vitro* by concentrations of chloramphenicol and aureomycin of 10 γ/ml and completely inhibited from growing by concentrations of 1,000 γ/ml. The concentrations required for appreciable inhibition of growth of cells are somewhat in excess of those which are bacteriostatic for most susceptible organisms but are within the range attained in the blood of treated patients. The amounts of the antibiotics required to produce toxic changes

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in the tissue culture cells are of the order sometimes reached in treating patients but those required for complete suppression of growth are well beyond clinical experience.

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Production of Achondroplasia in the Chick Embryo with Thallium.*

(17646)

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(Introduced by C. P. Rhoads.)

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The "4-amino" derivatives of folic acid have occasionally produced alopecia in man(1). Since thallium is also a systemically-acting depilatory(2,3), is was of interest to determine whether these two agents had other similar biological effects. The "4-amino" derivatives of folic acid produced a marked inhibition of growth of the chick embryo(4). It was, therefore, decided to study the effects of thallium on the same system.

Methods. Fertile White Leghorn eggs were obtained from a commercial source. The eggs were incubated at 38°C and 85% relative humidity. Thallium sulfate was made up in distilled water as a solution containing 10 mg/cc. This solution (the usual volume was 0.05 to 0.20 cc) was injected into the yolk sac of the 4 day embryo, except for a few experiments using 2, 10 and 12 day embryos. The eggs were candled daily, and most of the embryos were sacrificed at 18 to 21 days of age. Some of the embryos were cleared and their skeletons stained with alizarin red S by the method described by Couch, *et al.*(5). The protective value of manganese sulfate, riboflavin, biotin and nicotinamide against thallium was examined by injecting each of these agents separately, but simultaneously, with thallium sulfate into the yolk sac of the 4 day embryo.

Results. The acute (0-4 days after injection) LD_{50} of thallium sulfate for the 4 day embryo is about 1.3 mg/egg. Most of the embryos surviving the acute lethal effect of 1 mg/egg lived to 18 to 22 days of incubation, although none hatched (Table I). Thallium produced a severe and consistent picture of achondroplasia in the chick embryo, which was evident in embryos sacrificed or dying at 10 days of incubation or later. At the larger doses of thallium (1 to 2 mg/egg), achondroplasia was associated with a moderate to severe degree of growth inhibition. At the smaller dose of 0.5 mg/egg, growth inhibition was negligible, although a severe achondroplasia was frequently present. The gross appearance of the affected embryos is shown in Fig. 1. The embryos injected at 4 days of incubation showed a marked degree of achondroplasia, whereas those treated after 10 to 12 days of incubation showed a less severe but definite inhibition in the growth of the extremities. The severely achondroplastic embryos (treated at 4 days) showed very short limbs with feet that were of normal size

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* This study was aided by grants from the National Cancer Institute, National Institute of Health, United States Public Health Service, and the American Cancer Society.

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TABLE I.

Toxicity of Thallium Sulfate to 4-Day-Old Chick Embryo, and Incidence of Achondroplasia in Surviving Chick Embryos.

Dose, mg/egg	No. embryos	Acute toxicity (0-4 days after injection)	Mortality of embryos (death, or sacrifice ( ). Age of embryos, days)													Incidence achondroplasia, %
			9	10	11	12	13	14	15	16	17	18	19	20	21	
2.0	16	14					(2)									100
1.0	64	15			1	3(5)	(4)	1		(3)		3(6)	1	1	6(15)	92
0.5	15	4										(3)			6(2)	45*
0.2	5	1	2		(1)							(1)				0

* These embryos were within normal weight limits, and the achondroplasia was less severe than that occurring at 1 mg/egg.

or larger, principally due to edema, since the bones were small, parrot beak, thick trunk and a small head. On gross inspection of the head, the eyes appeared disproportionately small and the brain was small and seemed less solid than normal, particularly the cerebellum, which was atrophied. The abnormalities in eye and brain development were presumably due to compression of these organs due to the inhibition in skull growth. The feathers appeared to be well developed.

The normal and achondroplastic specimens, shown in Fig. 2, clearly show the defect in bone growth. The bones all appear to be present, but they are much shorter than normal, and the tibiae and femora are strikingly curved. Thallium sulfate did not produce achondroplasia when given at the maximum tolerated dose to the 2 day old embryo. However, the LD_{50} was estimated to be about 0.3 mg/egg, which is less than the amount of thallium sulfate necessary to consistently produce achondroplasia at 4 days.

The acute LD_{50} of thallium sulfate in the 10 to 12 day embryos (0 to 4 days after injection) was 2 mg/egg. The embryos surviving to hatching showed shortening of their extremities (Fig. 1) but the long bones of the legs were not curved, as seen on x-ray examination. Achondroplasia was produced by thallium nitrate at doses similar to those of thallium sulfate; in fact, the former preparation seemed to be more selective than thallium sulfate in its achondroplastic as compared to its lethal effect on the embryo. Ferrous sulfate and zinc sulfate were not toxic when injected into the yolk sac of the 4 day embryo at 5 mg/egg. These data indicate that the sulfate ion is not responsible or essential for the occurrence of achondroplasia.

Because of the reports that manganese(6,7), biotin(5,8) and riboflavin(9) deficiencies in the egg, and the injection of a nicotinic acid

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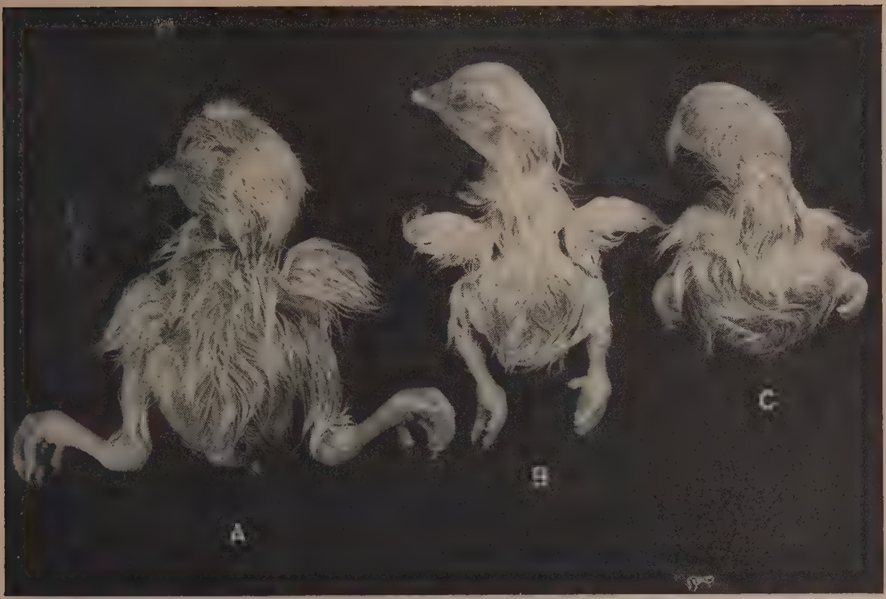

FIG. 1.

Gross appearance of chick embryos sacrificed at 21 days, immediately before hatching. A. Normal embryo. B. Embryo injected with 1.8 mg thallium sulfate at 12 days incubation. The skull and beak are not remarkable, but the extremities are considerably shortened. C. Embryo injected with 1 mg thallium sulfate at 4 days. This shows the characteristic picture of parrot beak, small head and eyes, short trunk, short extremities and feet of approximately normal size. There is over-all stunting of this embryo.

antagonist, 3-acetylpyridine(10) produce some form of achondroplasia in the chick embryo, the protective values of these agents in the chick embryo were tested against thallium. Four day embryos, treated with 1 mg/egg of thallium sulfate, were injected at the same time with either 5 mg manganese sulfate, 5 mg nicotinamide, 2 and 5 mg of biotin or 1 mg of water-soluble riboflavin (Lederle). The addition of thallium and one of these agents near their maximum tolerated doses caused a large percentage of early deaths in the embryos, but 3 to 5 embryos survived to 18 to 22 days in each group, and these all showed the characteristic and unmodified picture of "thallium" achondroplasia.

Discussion. Thallium has been found to exert a highly specific effect on the development of the chick embryo, by producing a severe degree of generalized achondroplasia. This effect does not occur if thallium is injected at the 2nd day of incubation, presumably because thallium is lethal to the 2 day embryo at a dose below that necessary to produce achondroplasia. At 4 days and later, the injection of thallium will interfere with bone development, the severity of the effect being related to the stage of embryonic development at the time of treatment. The localization of thallium in the chick embryo, the histology of the abnormal skeleton and changes in the embryo secondary to the failure of skeletal development will be the subjects of further studies. The achondroplasia produced by thallium appears to be similar to that described in hereditary chondrodystrophy in the chick embryo, and this has recently been analyzed as due to an autosomal recessive gene by Lamoreux(11). In the severe cases, parrot beak and curved tibiae

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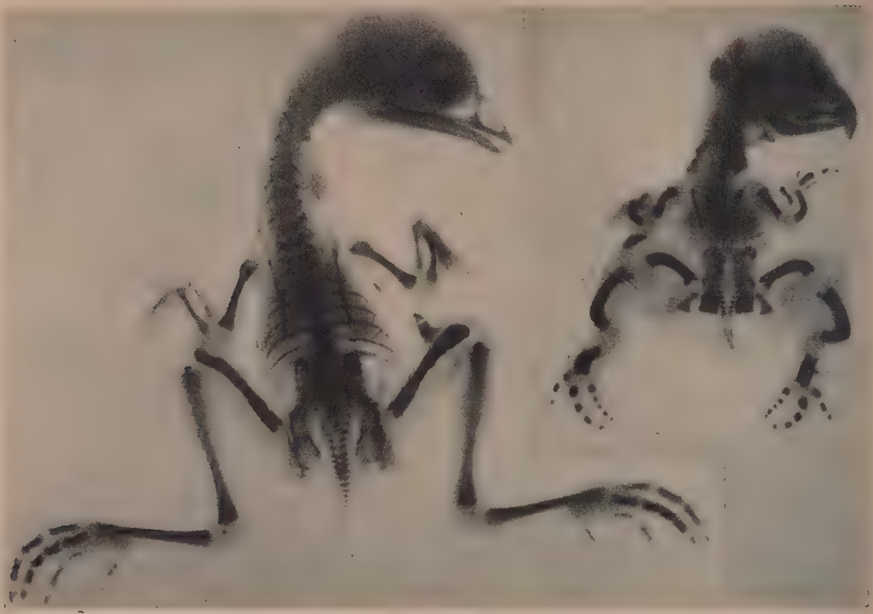

FIG. 2.

Skeletal structure of control and thallium-treated (1 mg/egg injected at 4 days) 21-day-old chick embryos. The diminution of skeletal growth is marked, and the curved femur and tibia are evident.

were present, and the embryos rarely hatched. While some of the embryos treated with thallium resembled the photographs published by Lamoreux(11), thallium can produce a more severe degree of achondroplasia. It is of great interest that a chemical can simulate a genetic abnormality, believed to be due to a single gene. Couch, *et al.*(5) and Cravens, *et al.*(8) have observed a high incidence of chondrodystrophy in the embryos from hens placed on a biotin-deficient diet, and this defect was prevented by injecting the egg with biotin. These embryos, as shown in published photographs(5,8), are also similar to the thallium-treated embryos. The sporadic occurrence of chondrodystrophy similar in type to the above—and the first reported instance of chondrodystrophy in the chick embryo—was described by Landauer and Dunn(12).

Micromelia and beak deformities have also been produced by the injection of insulin into the yolk sac of the 4 and 5 day old chick embryo(13), by sulfanilamide(14), by the nicotinic acid antagonist 3-acetylpyridine(10) and by the production of a riboflavin deficiency in the egg(9). Landauer(15) and Zwilling(16) have suggested that the micromelic action of insulin may be related to its hypoglycemic activity in the embryo, and this effect can be prevented by the administration of nicotinamide at the same time as the insulin. The micromelic effect of 3-acetylpyridine may also be related to this mechanism, and it is also of interest that the micromelia produced by sulfanilamide, although not related to hypoglycemia, is prevented by nicotinamide(16). Manganese deficiency in the egg also produces a type of chondrodystrophy, but the tibiae are not curved and the embryos show marked edema and retarded down development. The failure of manganese, nicotina-

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mide, biotin and riboflavin to prevent the achondroplastic effect of thallium, would suggest that thallium has a separate mechanism of action, although it is possible, on a more complete analysis, that some of these agents may act ultimately on the same site in affecting bone development.

Along with the genetic and nutritional deficiency technics for producing achondroplasia, thallium is now demonstrated to act in a highly consistent manner to produce a severe and uniform defect in the osseous development of the chick embryo.

Summary. Thallium sulfate produces a consistent and severe degree of achondroplasia in the chick embryo when injected on the fourth day of incubation. This abnormality appears to be similar to the genetic, and to some of the nutritional deficiency, types of chondrodystrophy. Thallium induced achondroplasia is not prevented by treating the embryo with manganese, biotin, nicotinamide or riboflavin, deficiencies of which have been reported to result in some type of chondrodystrophy in the chick embryo.

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Anesthetic Effects in Mice and Dogs of Some Unsaturated Hydrocarbons and Carbon-Oxygen Ring Compounds. (17647)

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Previous reports of anesthesia with butylene stated that the butylene used was a mixture of isomers(1) or that it was probably chiefly ethyl ethylene(2). Effects observed using butylene could not therefore be attributed to any one isomer. The recent development of efficient distillation procedures has made possible the separation of various hydrocarbons with a purity hitherto unattainable. This improvement has permitted a more definitive attempt to find relationships between chemical constitution and physiological activity.

Methods. The methods used have been described(3). Essentially the experiments were carried out in a large jar equipped through the rubber stopper with apparatus that provided for introduction of known quantities of oxygen or anesthetic compound while maintaining atmospheric pressure. Carbon dioxide was absorbed by soda lime. The oxygen concentration was kept at 21%. The experiments were carried out between 25 and 27° C.

Properties of the compounds used are given in Table I.

In order to standardize results the experiments were limited to concentrations of anesthetic agents that would afford surgical anesthesia within 10 minutes. Experiments were terminated after 20 minutes of anesthesia. Surgical anesthesia was accepted as the condition in which the righting reflex had been lost and in which the animals failed to respond to pinching a leg against the side of the jar with a stirring rod. At least 64 mice were used to determine each percentage value given in Table I. Probit analysis of results(4) was used in order to minimize the number of animals necessary from which to obtain statistically reliable values, for several of the materials tested were available in small quantities. To test the degree of sensitization of

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TABLE I.
Anesthetic Effect in Mice and Dogs of Some Unsaturated Hydrocarbons and Carbon-Oxygen Ring Compounds.

			% in atmosphere for		
Compound	Purity, %	Boiling point, °C	Surgical anesthesia	Respiratory arrest	Anesthetic index
Butene-2, cis, H.B.	96.18*	4	17.2	25.5	1.5
Butene-2, trans, L.B.	98.92*	1	18.7	21.0	1.1
Butene-1	99.88†	—6	22.7	27.2	1.2
Isobutylene	99.44*	—7	19.8	32.0	1.6
Butadiene-1,3	99.87*	—4	17.3	25.7	1.5
Pentene-1	99.34*	30	8.6	9.7	1.1
Propyne	97 plus	—105	Indeterminate	18.7	—
Butyne-1	97 plus	18	10.8	19.5	1.8
Butyne-2	97 plus	29	4.1	4.6	1.1
Pentyne-1	97 plus	40	6.9	14.1	2.0
Pentyne-2	97 plus	56	5.2	9.3	1.8
Heptadiyne-1,6	97 plus	108	2.0	4.1	2.05
Cyclopentene	99.76*	45	5.0	7.7	1.5
Cyclohexene	98	83-85	2.9	6.0	2.1
Trimethylene oxide		46.7-47.7	13.4	20.0	1.5
Pentamethylene oxide (Tetrahydropyran)	99	88	4.67	4.74	1.02
Dihydropyran	95	86	2.6	4.3	1.7
Dioxolane-1,3		74	14.3	19.3	1.3
2-Methyl dioxolane-1,3		81	11.4	17.1	1.6
4-Methyl dioxolane-1,3		85	12.8	14.6	1.1

* National Bureau of Standards, freezing point method.
† National Bureau of Standards, mass spectrometer method.

cardiac tissue to epinephrine by anesthetic agents, dogs were anesthetized and epinephrine was injected according to the technique of Meek, *et al.*(5).

Results. Induction of anesthesia with members of the ethylene series was accompanied by tachypnea and mild tremors of legs and head. A few tremors of hind legs were seen during anesthesia. Sleep was generally quiet but the mice were not well relaxed. Recovery was characterized by wild excitement lasting from 15 to 30 seconds. Induction with members of the acetylene series produced a greater state of agitation than in the ethylene series. Stimulation during induction with propyne was followed by convulsive movements. Continuous tremors and tonus of the tail appeared and were so extensive that one could not tell when surgical anesthesia was obtained. Surgical anesthesia with other acetylenic substances was not quiet, one or more legs moving constantly. Cyclopentene and cyclohexene produced only mild excitement during induction, and surgical anesthesia was quiet with the mice well relaxed. When respiratory arrest occurred it was preceded by convulsive movements. Induction was slow with all the carbon-oxygen ring compounds except di- and tetrahydropyran (pentamethylene oxide). With all this series induction was characterized by depression without stimulation of the nervous system.

Four mice anesthetized for 10 minutes with trimethylene oxide required 68 minutes for recovery. On the day following anesthesia all the animals that had been exposed were found dead. Sections were made of their brain, heart, lung, liver and kidney tissues. Congestion and acute hyperemia of the lungs were demonstrated microscopically.

With the exception of the mice inhaling trimethylene oxide, all the animals that had been anesthetized with the 20 compounds tested appeared healthy thereafter for a period of at least 2 weeks. With all the compounds tested, respiratory arrest preceded circulatory failure.

Electrocardiograms were made during injection of epinephrine when dogs were anesthetized with each of the following: butene-1;

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5. Meek, W. J., Hathaway, H. R., and Orth, O. S., *J. Pharm. and Exp. Therap.*, 1937, v61, 240.

cis butene-2; isobutylene; pentene-1; butadiene-1,3; cyclopentene; cyclohexene; propyne; and butyne-1. Arrhythmias were produced with each agent. The electrocardiograms using propyne were so abnormal before injection of epinephrine that it was difficult to say whether epinephrine caused a change. The arrhythmias were least significant with isobutylene, for in 4 dogs this substance produced only mild tachycardia and minor voltage changes. Results similar to those found here have been reported for butyne-1, cyclopentene, and cis trans butene-2 by Carr and Krantz(6).

Discussion. Three compounds gave anesthetic indices of 2 or higher: pentyne-1, cyclohexene, and heptadiyne-1,6. The disagreeable odor and the central nervous signs including tremors and convulsions eliminated pentyne-1 from clinical consideration. Convulsive movements were observed with cyclohexene just prior to respiratory arrest, which in addition to its odor and low volatility also eliminated its clinical consideration. Heptadiyne-1,6 had a strong odor of acetylene and was difficult to volatilize.

The concept that narcotic potency increases with molecular weight(7) was generally supported. However, butyne-2 required 4.1% while pentyne-2 took 5.2% concentration for surgical anesthesia. Other factors than concentration may have been responsible for this discrepancy, for both substances were powerful nervous system stimulants. The results also support the concept that anesthetic potency increases with degree of unsaturation(7). The outstanding observation of these comparative studies was that the position of unsaturation in the molecule appeared to have a substantial effect on the concentration required for both anesthesia and respiratory arrest, and on the degree of central nervous stimulation. Compounds with unsaturation in the 2 position were definitely more potent than the corresponding compounds with unsaturation at the end of the chain. This was true with both the ethylene and acetylene series of compounds.

The carbon-oxygen ring compounds were toxic rather than being anesthetic agents. Henderson(8) noted congestion and mottling of lungs after his mice had been exposed to tetrahydrofuran. Stehle(9) found universal hyperemia of parenchymatous organs after giving ethylene oxide. Trimethylene oxide produced congestion and mottling in the lungs of mice examined here. Mice that had been exposed to tetrahydropyran did not show these changes.

Summary. 1. Anesthesia was induced in mice with 20 different compounds which were highly purified unsaturated hydrocarbons and carbon-oxygen ring compounds. Concentrations required for surgical anesthesia and for respiratory arrest were measured.

2. Compounds with unsaturation in the 2 position in either the ethylene or acetylene series exhibited greater physiological activity than did corresponding compounds having unsaturation in the terminal position.

3. Trimethylene oxide and 5 and 6 membered carbon-oxygen ring compounds were toxic.

4. Where sufficient agent was available, sensitization of cardiac tissue of anesthetized dogs to epinephrine was determined. Irregularities of cardiac rhythm of at least moderate severity were produced with all 9 agents used except isobutylene which caused only mild tachycardia and minor voltage changes.

The author would like to express his thanks to: Dr. E. G. Gross of the Department of Pharmacology, State University of Iowa, for the use of facilities which made these experiments possible; the Phillips Petroleum Company of Bartlesville, Okla., which generously supplied the ethylenic hydrocarbons; the Farchan Research Laboratories of Cleveland, Ohio, who kindly furnished the acetylenic hydrocarbons; Dr. Ralph Shriner and Mr. Ralph Steinle of the Department of Chemistry, State University of Iowa, for preparation of the trimethylene oxide; the Dow Chemical Company of Midland, Mich., for the cyclohexene; the Carbide and Carbon Chemical Corporation of New

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6. Carr, C. J., and Krantz, J. C., Jr., *Fed. Proc.*, 1949, v8, 279.

7. Adriani, J., *The Pharmacology of Anesthetic Drugs*, C. Thomas, Springfield, 1941.

8. Henderson, V. E., and Smith, A. H. R., *J. Pharm. and Exp. Therap.*, 1936, v57, 394.

9. Stehle, R. L., Bourne, W., and Lozinsky, E., *Arch. f. exp. Path., u. Pharm.*, 1924, v104, 82.

York City, who supplied the dioxolanes; the E. I. du Pont de Nemours Company of Wilmington, Del., for the dihydropyran and tetrahydropyran; and to Dr. Robert Stickler of the Department of Surgery, State University of Iowa, who courteously prepared and examined microscopic sections of the tissues.

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Anatomic Changes Produced in Mice Treated with Excessive Doses of Cortisone. (17648)

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Hench, Kendall, Slocumb, Polley, Barnes and Smith(1,2) reported remarkable effects of Cortisone in patients with rheumatoid arthritis and rheumatic fever. These findings suggested the study of effects of large doses of Cortisone in animals. Accordingly, a dose equivalent to 50 times the amount used for humans was administered subcutaneously to mice. The limited quantity of Cortisone available sharply restricted our dose range and the number of animals treated. As additional amounts of Cortisone become available, we plan to use varying doses and different species.

Method. Two groups of mice were used. A) Nineteen (male and female) immature mice (21 to 30 days old) received daily subcutaneous injections of 1.25 mg Cortisone, suspended in 0.1 cc saline, for 6 successive days, unless they died or were sacrificed earlier as indicated below. Twenty comparable controls received 0.1 cc saline only on the same days by the same route. B) Seventeen mature (male and female) mice weighing 19-26 g received daily subcutaneous injections of 2.5 mg Cortisone suspended in 0.1 cc saline. They were thus injected for 9 days, unless they died or were sacrificed earlier as indicated below. Twenty comparable controls received 0.1 cc saline only for the same time by the same route. The mice were kept in an air conditioned room the temperature of which ranged from 79-85°F. They received water and Rockland Farm pellets *ad lib.*

Results. Of the 19 immature (21 to 30 day old) mice, one treated mouse with its corresponding control was sacrificed on the 2nd, 3rd, 4th, 7th, 9th, 10th, and 11th day after initiation of treatment. One died on the 5th day, one on the 6th day, five on the 8th day, 2 on the 9th day and one each on the 11th, 12th and 15th day. Of the 17 mature mice, one treated mouse and its corresponding control, was sacrificed on the 3rd, 9th and 17th day, after initiation of treatment, and one mouse died on the 5th, 7th, 9th, 16th, 17th and 19th day. Three died on the 8th, day, 3 on the 11th day, and 2 on the 13th day.

Some of the treated animals gained weight for one to 2 days and then lost weight; others lost weight from the outset. Those animals which died showed a precipitous weight loss during their last days. The average weight change of the immature group is shown in Fig. 1. This shows the loss of weight in the treated animals in contrast to the controls which gained weight. The results were essentially alike in the mature mice. Loss of body weight following Cortisone administration was reported by Kendall(3) and Kocha-

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[*] From the Joseph and Helen Yeamans Levy Foundation, established in memory of their daughter, Miriam Levy Finn, and the laboratories of Beth Israel Hospital, New York City.

1. Hench, P. S., Kendall, E. C., Slocumb, C. H., and Polley, H. F., *Proc. Staff Meet. of the Mayo Clinic*, 1949, v24, 181.

2. Hench, P. S., Slocumb, C. H., Barnes, A. R., Smith, H. L., Polley, H. F., and Kendall, E. C., *Proc. Staff Meet. of the Mayo Clinic*, 1949, v24, 277.

3. Kendall, E. C., *Conference on Metabolic Aspects of Convalescence Including Bone and Wound Healing*, 10th Meeting, 1945, p. 87.

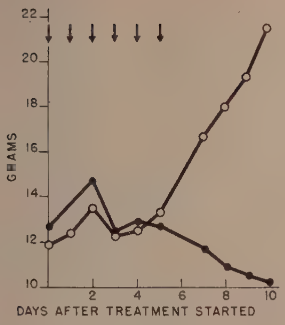

Fig. 1.
Weight Chart of Immature (21-30-day-old) Mice.
● Cortisone treated.
○ Control.
↓ Injection of 1.25 mg Cortisone or 0.1 cc saline.

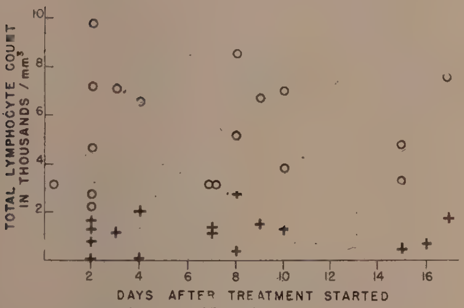

Fig. 2.
Total Lymphocytes in Peripheral Blood of Mice.
+ Cortisone treated.
○ Control.
Each point indicates a single determination on one mouse.

kian(4).

Diarrhea was occasionally encountered in the treated animals, but not in the controls.

The lymphocytes in the peripheral blood of the Cortisone treated animals fell below those of the controls and remained low for at least 17 days (Fig. 2). With the decrease in lymphocytes there was a corresponding increase in polymorphonuclear leucocytes[†].

In one cage with 4 Cortisone treated immature mice, a leaking water bottle wet the cage and its contents for about 12 hours. The mice became wet, cold and clammy. Despite drying, removal to a warm room and special care, all 4 mice died within 48 hours. This was repeated with 4 non-treated immature mice; no ill effects were noted.

Post Mortem Findings. After several days, the treated mice at autopsy showed loss of body fat. The abdominal fat was decreased considerably and the hibernating fat bodies were smaller than in the controls. In some instances, foci of necrosis and calcification were found in the hibernating bodies. Kendall(3) found very little excess of body fat in Cortisone-treated mice. Table I gives the average weight and range of 11 organs in the Cortisone treated and in the control immature mice. It shows the striking decrease in weights of the thymus and spleen, the less striking decrease in the salivary glands, hibernating fat bodies, and adrenal, and the slight decrease in the pituitary.

The testes, seminal vesicles and prostate were also smaller than in the controls, and the ovaries appeared smaller.

A few of the histological findings are mentioned below. A detailed histologic report will follow. The thyroid was in the resting phase with an abundance or even an excess of deeply staining eosinophilic colloid. The liver was usually small, but occasionally contained necrotic foci, in which latter instances there was enlargement of the organ. The diminution in size of the adrenal was due almost entirely to narrowing of the cortex. The testes showed retarded development. None of these changes were observed in the controls. Similar changes were likewise found in the mature mice after Cortisone administration. However, the testes showed varying degrees of suppression of spermatogenesis with appearance of giant cells. The latter apparently were due to fusion of secondary spermatocytes.

Granulomatous nodules in the liver, kidney, and spleen (but not at the site of injection) were found in 8 treated animals, and not in the controls. From these, *Corynebacterium pseudotuberculosis murium* was cultured.

Discussion. Selye(5) has written extensively on the systemic reaction produced after administering various drugs or subjecting animals to physical stress such as excess exercise or

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4. Kochakian, C. D., *ibid.*, 6th Meeting, 1944, p. 13.

[†] This will be reported in detail with Drs. H. Quitner, L. Sussman, and N. Wald.

TABLE I.
Comparison of Body and Organ Weights in Immature Mice Treated with Cortisone and in Saline Controls. The average of the body weight and 11 organs are given for both Cortisone treated and control mice. It shows the striking changes in some organs while others are comparatively unaffected.

	Controls (5 mice)		Cortisone-treated (9 mice)	
	Range, mg	Avg, mg	Range, mg	Avg, mg
Pituitary	0.85-1.35	1.15	0.60-1.83	1.03
Salivary Gland	132-212	168	40-154	102
Thymus	26.2-73	48.1	0.9-17.1	8.05
Heart	82-100	89	65-94	81
Spleen	142-185	164	32-78	51
Pancreas	70-95	79	28-100	67
Liver	905-1929	1180	480-1246	878
Hibernating Gland (interscapular)	73-175	128	18-220	59
Hibernating Gland (prescapular)	44-92	71	18-61	34
Adrenal	1.80-2.90	2.34	1.10-1.88	1.56
Kidneys	247-360	289	222-338	274
Body wt beginning of exper.	9-14 g	12.0 g	8.5-13 g	11.3 g
Body wt death of animal	12.5-18 g	14.8 g	7.0-13.0 g	10.6 g

exposure to cold. From our observations, it is apparent that large doses of Cortisone produced anatomic changes which in many respects were similar to those described by Selye after administration of "alarm producing agents". The assumption is that these "alarm producing agents" cause the liberation of pituitary adrenocorticotropic hormone; this, in turn, increases the activity of the adrenal cortex, causing hypertrophy with concomitant liberation of adrenal "corticoids". But in the Cortisone-treated animals, there was an atrophy of the adrenal cortex. This suggests that Cortisone may be one of the hormones responsible for, and capable of eliciting directly the "alarm reaction" without the intermediary of the pituitary or of the adrenal cortex. The administration of an overwhelming amount of this hormone in the body eliminated the need for any further cortical substance, therefore obviating the necessity for an enlargement of the adrenals. The atrophy of the adrenal cortex may also be explained on the basis of compensatory atrophy of the adrenals similar to that found by Selye with desoxycorticosterone(5).

The slight diminution in size of the pituitary in the treated mice suggests the possibility of an inhibiting effect of large doses of Cortisone upon this organ, with consequent diminution in ACTH production. Such diminution of the ACTH may also play a role in decreasing the size of the adrenal(5).

The lymphopenia produced after the administration of Cortisone corresponds to the findings of White and Dougherty after the administration of ACTH(6). These authors believed the lymphopenia to be due to increased adrenal cortical secretion.

The diminished ability to withstand exposure to wetting may be explained on the basis of Selye's(5) observation that animals receiving repeated injections of desoxycorticosterone develop adrenal atrophy. Such animals "respond somewhat like adrenalectomized animals, and exhibit low general resistance and hypoglycemia during stress". The mechanism of the reaction may be similar to that in thiouracil treated rats, reported by Zarrow and Money(7), in which the hypothyroid rats developed adrenal atrophy.

The frequency of *Corynebacterium pseudotuberculosis murium* infection in the treated animals may depend upon a loss of immunity (8). With disappearance of the lymphocytes and their contained antibodies, there probably

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5. Selye, Hans, *J. Clin. Endocrinology*, 1946, v6, 117; *Endocrinology*, 1937, v21, 169.

6. White, A., and Dougherty, T. F., PROC. SOC. EXP. BIOL. AND MED., 1944, v56, 26.

7. Zarrow, M. X., and Money, W. L., *Endocrinology*, 1949, v44, 345.

is depletion of immune bodies with resulting insufficient antibody for fixing the antigen.

Summary. 1. The administration to mice of Cortisone in doses approximately 50 times the dose for humans produced a striking lymphopenia, loss in body weight, atrophy of thymus and spleen, and diminution in size of adrenal cortex, salivary glands, pituitary, and hibernating fat bodies. A number of histologic changes are briefly described.

2. The anatomic findings correspond to those of the "alarm and adaptation" phenomena of Selye except for atrophy of the adrenal cortex in place of hypertrophy. Cortisone *per se* may produce some of the morphologic changes associated with the "alarm reaction."

3. Administration of these excessively large doses of Cortisone may produce exhaustion of protective mechanisms, including antibody formation.

We should like to express our appreciation to Drs. Philip Hench and James Carlisle for their interest in these investigations and for making available the Cortisone used in these studies.

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8. Dougherty, T. F., Chase, J. H., and White, A., PROC. SOC. EXP. BIOL. AND MED., 1945, v58, 135.

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Weight Variations of Adrenalectomized Frog Muscles in Different Concentrations of Ringer's Solutions.* (17649)

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It has been shown that following adrenalectomy, body water tends to shift into certain tissues and cells (skeletal muscles of rats(1) and frogs(2), and red cells of dogs(3), cats(4) and rats(5)). According to Winter and Hartman(6), not confirmed by Ponder and Gaunt(7), excised skeletal muscles of adrenalectomized rats shift water in a given hypotonic solution at a greater rate than controls probably because of some alteration in the supposed plasma membranes of the myofibers. Some unrecognized variable, *viz.*, diet, water balance, etc., may be the basis for the observed discrepancy. We reinvestigated(8) the problem studied by the foregoing workers using their experimental technic on skeletal muscles of apparently more favorable material. The frog was selected for the following reasons: 1) nutrition is simplified, assuming that a plentiful supply of testicular fat at autopsy is indicative of at least a "basal" state; 2) imbibition of water is potentially continuous from an aqueous environment(9); 3) isolated muscles of the frog immersed in solutions at room temperature are more within their thermal biokinetic range than are muscles of poikilotherms in solutions from 15 to 20°C lower than their body temperature(10).

Methods. Male *Rana pipiens* (fall and win-

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* This investigation was aided by a grant from the Comly-Coleman Research Fund of the Ohio State University.

1. Crismon, J. M., and Field, J., 2nd, *Am. J. Physiol.*, 1940, v130, 231.

2. Angerer, C. A., *Am. Physiol. Soc. Proc.* 1942, v1, 3.

3. Harrop, G. A., *Bull. Johns Hopkins Hosp.*, 1936, v59, 11.

4. Hegnauer, A. H., and Robinson, E. J., *J. Biol. Chem.*, 1936, v116, 769.

5. Gonzalez-Q., J., and Angerer, C. A., *Am. J. Physiol.*, 1947, v149, 502.

6. Winter, C. A., and Hartman, F. A., PROC. SOC. EXP. BIOL. AND MED., 1933, v31, 201.

7. Ponder, E., and Gaunt, R., PROC. SOC. EXP. BIOL. AND MED., 1934, v32, 202.

8. Angerer, C. A., and Angerer, H. H., *Am. J. Physiol.*, 1941, v133, 197.

9. Adolph, E. F., *Physiological Regulations*, p. 110, The Jaques Cattell Press, Lancaster, Pa., 1943.

10. Belehradek, J., *Temperature and Living Matter*, p. 136, Gebruder Borntraeger, Berlin, 1935.

ter stock) weighing 20 to 30 g were divided into 4 groups (between 15 and 18 frogs/group): 1) *"controlled" adrenalectomized,* 2) *"uncontrolled" adrenalectomized,* 3) *renal damaged* (controls) and 4) *unoperated* (controls) frogs. The methods of dividing the frogs into groups, of adrenalectomy and of determining adrenal insufficiency have been previously discussed(11).

Ringer's solution(12) was prepared in 18-liter lots; the pH was adjusted to 7.4 with $NaHCO_3$. Pyrex beakers, serving individual muscles were filled with 200 cc of one of the following Ringer concentrations: 2X or 3X normal (hypertonic); normal (isosmotic); 75, 50, or 25% of normal strength (hypotonic). The specific muscles studied were the ileofibularis, sartorius and semitendinosus. A pair of specific muscles taken from left and right legs of the same frog give a consistent error for the difference in their individual weights as obtained before and after immersion in a solution of given osmotic strength. Consequently, an equal number of specific muscles excised from an equal number of left and right legs of different frogs were used to produce a mean value (point) on the accompanying graph. Upon excision a muscle was carefully blotted of excessive body fluid, rapidly weighed with a torsion balance, and immersed in a given strength of Ringer's solution at 14 ± 1°C. The time of immersion was recorded from the moment a muscle was placed in solution, and at the end of predetermined intervals (see figures) the muscle was weighed as described (elapsed time *ca.* 20-30 seconds). At the end of an experiment the muscle was returned to aerated normal Ringer's solution at *ca.* 5-7°C. Thereafter, tests were made periodically to determine the duration of survival of its irritability.

The means for any two groups of data were statistically compared. Hereafter, a comparison is considered significant when the difference between a given pair of means exceeds 3 times its probable error.

Results. Data obtained for any one of the 3 specific muscles following their immersion in Ringer's solution of given osmotic strength are relatively similar. Therefore, ileofibulares from the 4 frog groups immersed respectively in 50%, 2X and in normal Ringer's solution were chosen to summarize the data.

Hypotonic solutions. Data for muscles immersed in 50% Ringer's solution are presented in Fig. 1. An analysis of these curves shows that the rates of increase and the total gain in muscle weights are directly related. Further, the rates of increase and the total gain in weights of muscles from the 4 frog groups following their immersion in hypotonic (50%) Ringer's solution are greater for muscles from "controlled" adrenalectomized frogs than from either unoperated or renal damaged frogs. Similarly, muscles from the latter 2 frog groups give a greater increase in total weights and in rates of weight gain than muscles from "uncontrolled" adrenalectomized frogs. The irritabilities of muscles from unoperated and renal damaged frogs show a longer survival time than muscles from adrenalectomized frogs.

Hypertonic solutions. The rates of decrease and the total loss in weights following immersion of muscles from the 4 frog groups in hypertonic (2X) Ringer's solution are inversely related to the results obtained for the same muscles immersed in hypotonic (50%) Ringer's solution. A statistical analysis of these data shows that decrease in the rates of and the total loss in muscle weights are lowest for "controlled" adrenalectomized frogs, highest for "uncontrolled" adrenalectomized frogs, interposed are renal damaged and unoperated frogs. Incidentally, the latter 2 groups are without statistical significance throughout this report.

Isosmotic solutions. When the percentage changes in mean weights following the immersion of muscles from the 4 frog groups in normal Ringer's solution are compared (Fig. 2), it is found that muscles from the "controlled" adrenalectomized frogs and from unoperated and renal damaged frogs increase their weights; while muscles of "uncontrolled" adrenalectomized frogs lose weight.

Discussion. Weight changes of skeletal

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11. Angerer, C. A., and Angerer, H. H., PROC. SOC. EXP. BIOL. AND MED., 1949, v71, 661.

12. Bayliss, W. M., *Principles of General Physiology,* p. 211, Longmans, Green and Co., Ltd., London, 1927.

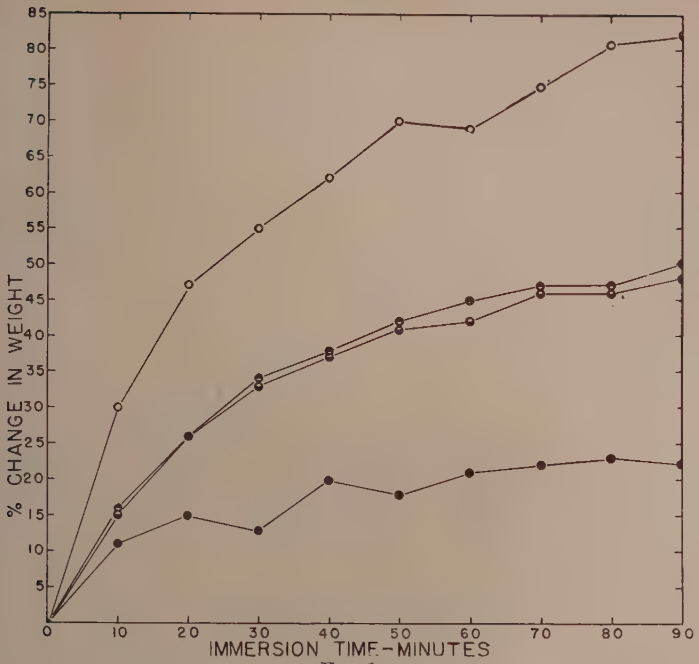

FIG. 1.

The percentage change in the mean weights of ileofibularis muscles excised from the following designated groups of frogs immersed in 50% Ringer's solution (ordinates) for varying periods of time in minutes (abscissae). Frog groups: "controlled" (open circle) and "uncontrolled" (solid circle), adrenalectomized frogs; and the unoperated (upper half solid circle) and renal damaged (lower half solid circle) frogs. Temp. 14 ± 1°C; pH 7.4.

muscles in solutions of various osmotic strengths indicate that they are not perfect osmometers. The evidence suggests "that the osmotic behavior is a reflection of forces relating to ion accumulating mechanisms and is not due to the existence of a discrete semipermeable membrane of fixed dimensions" (13). Until these forces are understood for the apparent osmotic behavior of "normal" muscles, it seems inadvisable to venture an interpretation of the osmotic behavior of muscles of adrenalectomized animals.

Muscles from "controlled" adrenalectomized frogs show higher rates of water shift on immersion in a given hypotonic solution than do muscles obtained from either renal damaged or unoperated frogs, (Fig. 1). These results parallel those of Winter and Hartman(6) for adrenalectomized rats. They differ in that the total amount of water shifted is a function and not independent of the experimental group from which the muscle is obtained. On the other hand, muscles from "uncontrolled" adrenalectomized frogs show lower rates of water shift and the total water shifted is less than for muscles from either renal damaged or unoperated frogs. These results simulate those of Ponder and Gaunt(7). The crucial variable accounting for the apparently divergent results obtained for the 2 groups of adrenalectomized frogs as well as for the two reports on adrenalectomized rats(6,7) is the difference in water load(14). It has been established that certain cells(3,4,5) and, more pertinently, skeletal

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13. Steinbach, H. B., *J. Cell. and Comp. Physiol.*, 1944, v24, 291.

14. Angerer, C. A., *Ohio J. Sc.*, 1950, in press.

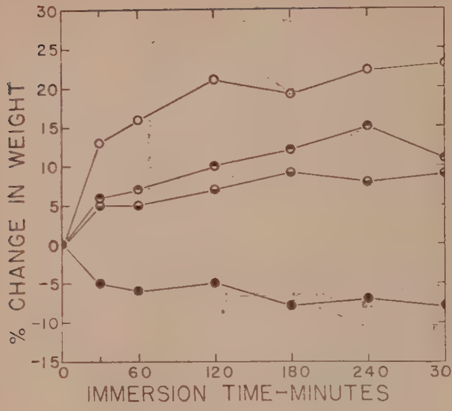

FIG. 2.

The percentage change in the mean weights of ileofibularis muscles excised from the following designated groups of frogs immersed in normal Ringer (ordinates) for varying periods of time in minutes (abscissae). Frog groups: "controlled" (open circle) and "uncontrolled" (solid circle) adrenalectomized frogs; and the unoperated (upper half solid circle) and renal damaged (lower half solid circle) frogs. Temp. 14 ± 1°C; pH 7.4.

muscles of rats(7,1) as well as of frogs(2) accrue a water load following adrenalectomy. The effect of the initial water load on weight change is dramatically shown when muscles are immersed in normal Ringer's solution. All muscles of the 4 frog groups behave "normally" in that they increase their weights(15) (*i.e.*, solution acts hypotonically), except for muscles of "uncontrolled" adrenalectomized frogs which decrease their weights (*i.e.*, solution acts hypertonically). These results on "uncontrolled" adrenalectomized frogs are in accord with those of Ponder and Gaunt on adrenalectomized rats, with one exception: although their data showed no statistical significance for weight changes in hypotonic solutions the present data do. This apparent inconsistency is probably explainable on the basis of the kind of animal employed. The frog given a copious aqueous environment continuously imbibes water through its integument(9), and in adrenal insufficiency increases its body (water) weight(16,14), whereas the rat is dependent on the physiologically little understood "drive" called thirst to aid in maintenance of body water, and in adrenal insufficiency decreases its body weight(17).

That the irritability of skeletal muscles of adrenalectomized frogs fail to survive as long as muscles from either renal damaged or unoperated frogs is to be anticipated from studies of direct stimulation of frog muscle with respect to isotonic contractions and work performance(16,18).

Summary. Ileofibularis, sartorius and semitendinosus muscles were obtained from 4 groups of grass frogs: 1) *"controlled" adrenalectomized;* 2) *"uncontrolled" adrenalectomized;* 3) *renal damaged* (controls); and 4) *unoperated* (controls) frogs. These muscles were studied with regard to changes in their initial weights following predetermined periods of immersion either in hypotonic, isosmotic or in hypertonic Ringer's solution. The rates of increase and the total gain in weights of muscles from the 4 frog groups following the immersion of the muscles in hypotonic (50%) Ringer's solution are highest for the "controlled adrenalectomized frogs, lowest for the "uncontrolled" adrenalectomized frogs. The same muscle groups when studied in hypertonic (2X) Ringer's solution give results inversely related to the foregoing values. In normal Ringer's solution, muscles from the "uncontrolled" adrenalectomized frogs lose water, those from "controlled" adrenalectomized, renal damaged and unoperated frogs behave normally by increasing their weights. These results are interpreted on the basis of the difference in the initial water loads of the skeletal muscle of the respective groups of frogs which are known to exist prior to experimental use.

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Technic for Measuring Cardiac Output Directly by Cannulation of the Pulmonary Artery. (17650)

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Cardiac output has been measured directly in the experimental animal by inserting flow meters into the aorta(1,2), or into the venae cavae(3,4). Such procedures, however, do not determine total cardiac output, since they fail to measure coronary flow. By measuring aortic plus coronary flow simultaneously, the total output of the heart can be obtained, but this requires the added operation of cannulating both coronary arteries successfully. Since these difficulties could be avoided if the pulmonary artery were cannulated and flow through it measured, a technic to do this in the dog has been devised and is presented here.

Under anesthesia and artificial respiration, the left side of the chest is opened (ribs 3 to 6 removed) and the pulmonary artery exposed. The artery is dissected free from its origin out onto the right and left branches about ¾ of an inch. After heparinization (10 mg per kilo), the right branch is ligated at its origin, cannulated peripherally, and cut free from the bifurcation (Fig. 1). A continuous vessel, long enough for cannulation, is now formed by the pulmonary artery and its left branch. This structure may be cannulated without bleeding by one of 2 procedures.

In the technic which has been most often successful, a shunt around the pulmonary artery is provided during cannulation. To do this, a pump, primed with compatible, donor blood, is connected between the right atrial appendage and the previously cannulated peripheral end of the right pulmonary artery (see dashed lines in Fig. 1). When ligature D is tightened just above the pulmonic valves, the main pulmonary artery and left branch can be cannulated, while the circulation is maintained via the pump-shunt system. The rotameter assembly unit is then attached to the cannula. Ligature D is released, the pump stopped, and flow reestablished through the pulmonary artery, rotameter, and left branch. The right pulmonary artery cannula is disconnected from the pump and joined to the outflow side of the rotameter. This reestablishes flow to the right lung. When stopcock C is closed, the output of the right heart passes through the rotameter and is distributed to both lungs. Zero flow levels may be recorded by opening stopcock C and closing clamps A and B.

To permit cannulation without hemorrhage, an alternative to the pump-shunt procedure has been used successfully in a few exploratory

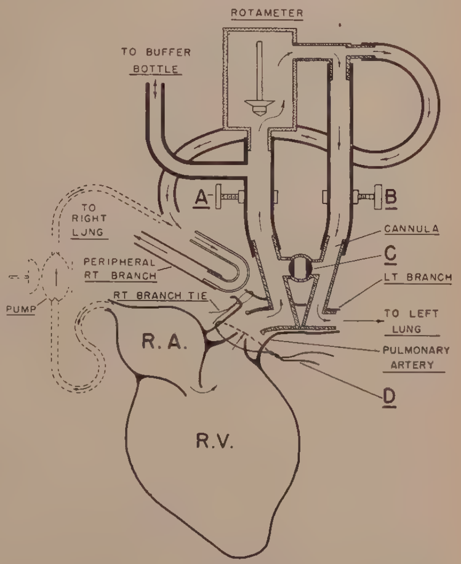

FIG. 1.
Description in text. Buffer bottle (not shown) is added to diminish oscillations of rotameter float.

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experiments. After the pulmonary artery and its branches have been freed and the right branch prepared as above, the main artery is occluded centrally, and the heart is fibrillated by means of an inductorium. A cannula, like that in Fig. 1, is quickly inserted into the artery and tied in place with the circulation at a standstill. The heart is then defibrillated with countershock, according to the method of Wiggers(5), and an active circulation is reestablished. The rotameter assembly unit is finally attached to the cannula. Success with this approach is limited by the time necessary for cannulation; for if fibrillation persists longer than 1 to 2 minutes, countershock alone is usually ineffective in terminating the fibrillation. Cardiac massage must then be combined with countershock to restore the heartbeat, and the reestablished circulation may not be adequate. However, if cannulation is completed within 1 to 1½ minutes, revival is usually easy and the beat is sufficient to maintain an adequate output.

The pulmonary artery has been cannulated successfully in 9 of 12 dogs, and flows recorded by the rotameter range from 40 to 100 cc per kilo.

Acknowledgment is made of the technical assistance given in these experiments by Edward M. Khouri, and F. H. Longino.

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Antagonistic Action of Certain Neurotropic Viruses Toward a Lymphoid Tumor in Chickens with Resulting Immunity.* (17651)

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Moore(1) reviewed the literature on the growth of viruses in tumors and reported the tumor-necrotizing action of the Russian Far East encephalitis virus on mouse sarcoma 180(2). In the report that follows, results are presented of experiments carried out with the Russian Spring-Summer encephalitis virus (RSS) in domestic chickens infected with a transplantable malignant lymphoid tumor (3,4). A number of other viral and rickettsial agents also were tested, results of which are recorded briefly.

Experimental tumor. The RPL-12 lymphoid tumor(5) originally described by Olson (6) was received through the kindness of Drs. B. R. Burmester, G. E. Cottral and B. Winton, Regional Poultry Research Laboratory, U. S. Department of Agriculture, East Lansing, Mich. Because on storage there is considerable loss of infectivity, freshly harvested tumors were used in each experiment. Tumors were removed in early stage of growth and homogenized with saline solution in a TenBroeck grinder(7). Unless otherwise stated, the infecting dose used was 0.25 ml of 1:1,000 suspension of tumor tissue injected into the pectoral muscle.

Viral and rickettsial inocula. The following viruses were used: mumps (Enders strain), swine influenza (Shope strain No. 15) in form of infected allantoic fluid from chick embryos; canine distemper (Lederle strain) as infected chorioallantoic membrane; Columbia-SK, Bwamba (M483 strain(8)), Semliki Forest (9), Ilheus encephalitis(10), Theiler's GD VII mouse encephalomyelitis(11), eastern

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* Our interest in virus-tumor antagonism was stimulated by personal communications from Dr. C. P. Rhoads, Director, Memorial Hospital, and the Sloan-Kettering Institute for Cancer Research, prior to the publication of Dr. Moore's results.

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TABLE I.
Relationship of Variations in Age of Host and Tumor to Response Following Injection of RSS Virus.

Age of tumor,* days	Age of bird, days	RSS treated† Survival ratio	RSS treated† % survival	Untreated controls Survival ratio	Untreated controls % survival
2	1	14/18	78	3/20	15
6-9	1-2	2/38	5	1/32	3
2-4	7	36/48	75	0/33	0
5-6	7	26/28	93	6/20	30
7-9	7	9/41	22	1/51	2
2-4	14	99/112	88	6/95	6
7-9	14	39/69	57	15/68	22
2-3	21	27/28	97	4/29	14
9	28	15/26	58	5/24	21

* The number of days elapsing between tumor inoculation and injection of RSS virus is termed "Age of Tumor."

† Virus inoculum consisted of 0.5 ml of 1:100 dilution of RSS-infected mouse brain.

equine encephalomyelitis as infected mouse brain suspensions; western equine encephalomyelitis as infected whole chick embryo; the Pitman-Moore No. 980 strain of rabies virus as a suspension of infected rabbit brain, the Flury strain(12,13) and Novisad strain(14) of rabies virus as saline suspensions of whole chick embryo. The RSS(15), West Nile (M-956 strain(16)), Japanese B encephalitis (Nakayama strain(17)), St. Louis encephalitis (Brown strain) and louping-ill viruses were used in form of infected mouse brain tissue suspensions. The LD$_{50}$ titers in intracerebrally injected 28-day-old mice were: RSS 10^{-5} to 10^{-6}; West Nile 10^{-8} to 10^{-9}; Japanese B encephalitis 10^{-5}; St. Louis encephalitis $10^{-4.5}$, and louping-ill $10^{-5.5}$. Rocky Mountain spotted fever (Montana R strain), North Queensland tick typhus (PHS strain), rickettsial pox (McCauley strain), boutonneuse fever (Moroccan strain No. 1), South African tick bite fever (Malish No. 7 strain), epidemic typhus (Breinl strain), murine typhus (Wilmington strain), *Lymphogranuloma venereum* (Nigg strain), feline pneumonitis (Baker strain), human pneumonitis (SF strain), and bovine encephalitis (McNutt strain) were used as infected yolk-sac tissue suspensions.

Chickens. All of the experiments were done with New Hampshire Red chicks. Since all lots of chicks tested were susceptible to the tumor, no attempt was made to obtain all birds from the same flock.

Relationship of variation in age of host and of tumor to response following RSS-virus inoculation. Since the results of a preliminary experiment indicated that RSS virus had some oncolytic action, and that the age of the bird may have some bearing on the outcome, additional birds ranging in age from 1 to 28 days were inoculated with tumor and 2 to 9 days later were injected in the tumor or tumor-inoculation site with 0.4 to 0.5 ml of 1:100 RSS-infected mouse brain suspension. It was observed that tumors continue to grow for a few days after infection with RSS virus and although frequently no difference in size can be seen for 6 to 7 days, the virus-treated

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TABLE II.
Extra-Tumor Inoculation of RSS Virus in 2-week-old Chicks.

Age of tumor, days	RSS-infected mouse brain* Treated	RSS-infected mouse brain* Untreated	6th or 8th passage RSS in tumor† Treated	6th or 8th passage RSS in tumor† Untreated
	Survival ratio		Survival ratio	
1	4/10	1/9		
2	7/8	1/9	15/20	1/19
3	7/10	1/9	10/10	1/10
4	6/10	1/9	20/20	1/19

* .5 ml of 1:100 RSS-infected mouse brain (mouse LD_{50} titer $10^{-5.5}$).
† .5 ml of 1:100 RSS-infected tumor tissue (mouse LD_{50} titer $10^{-5.5}$).

tumors tend to be less firm. In untreated chicks up to 2 weeks of age the tumors grow rapidly and most of the birds die within 8 days after the tumor becomes palpable, whereas tumors in older birds attain a greater relative size and are associated with a longer survival time. Many of the chicks treated 2 days after tumor inoculation did not develop palpable tumors, whereas most of those treated 4 days after tumor-inoculation did, but the tumors remained small and regressed completely within 5 to 10 days. Tumors occasionally failed to develop in chicks which were not treated. From the data presented in Table I it may be noted that regardless of the age of the birds, when RSS virus was introduced into the tumor site 2 to 4 days after inoculation a high percentage of the treated birds survived, but the higher survival rates among the untreated controls at the 2 to 4 week age levels suggest that an age-resistance factor may have some influence at this time.

Effect of mixed RSS virus and tumor inoculum. Since the survival ratios were much greater when RSS virus was introduced 2 to 4 days after tumor inoculation, it was of interest to determine what the effect would be when the inoculum consisted of a mixture of tumor suspension and RSS virus. One group of 9 chicks received 0.25 ml of a mixture containing 1:100 tumor suspension (10 times the usual infecting dose) and 1:100 dilution of RSS-infected mouse brain, which was held at 4° C for 1 hour prior to injection. All of the birds survived but on the 8th day 2 of them had small tumors which, however, regressed completely within the next 7 days. The second group of chicks was given a similar mixture except that the RSS infected mouse brain was diluted 1:10,000. Nine of the 10 birds survived, but 3 of the survivors showed palpable tumors which later regressed. Of the 15 control birds none survived.

Effect of mouse brain tissue on tumor growth. Two-week-old chicks were treated 3 days after tumor inoculation with 0.5 ml of 1:100 suspension of normal mouse brain tissue given into the tumor-injection site. Of the 29 so treated 7 survived (24%) while of the 19 tumor-bearing untreated control chicks 6 survived (31%). Further evidence that mouse brain tissue had no oncolytic action was provided by the failure of treatment with such tissue infected with other viruses. In all, 98 chicks were so treated and of these only 9 (9%) survived while of 95 in the untreated control groups 7 (7%) survived.

Effect of extra-tumor injection of virus. While the data in Table II show that inoculation with RSS-infected mouse brain suspensions into breast opposite to that of tumor inoculation saved a significant number of birds, it should be stated that most of the tumors became large before regressing, but treated chicks that died with tumors had a longer survival period than the untreated controls. Tumor-regression was likewise obtained when the inoculum comprised 6th or 8th passage RSS virus in the RPL-12 tumor†

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† The passages of RSS virus in RPL-12 tumors were made in chicks with palpable tumors by injecting into the side of the breast opposite the tumor 0.5 ml of 1:100 suspension of virus-infected tumor from the previous passage. Three or 4 days after the birds were infected with virus, the tumors were harvested, made to a 20% suspension in distilled water in a Waring blendor and stored at —30°C.

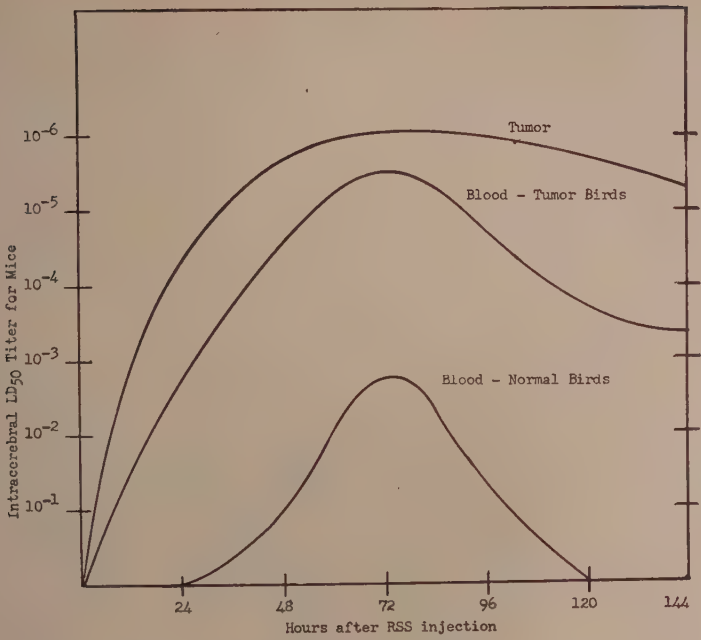

FIG. 1.

Concentration of RSS virus in blood of normal, and blood and tumor of tumor-bearing chicks at intervals following infection.

introduced into the side opposite to the tumor inoculation. A comparison of the data shown in Table II with those in Table I indicates that RSS virus obtained from tumors is as effective when given at a distance from the tumor as is RSS virus-infected mouse brain given into the tumor-inoculation site. That the difference is qualitative rather than quantitative is indicated by the fact that the mouse LD_{50} titers were the same for each type of inoculum.

RSS virus titer in the tumor. A group of 20 chicks with palpable tumors in the left pectoralis was given in the opposite breast 0.5 ml of 1:100 dilution of RSS-infected mouse brain suspension. At each successive 24-hour interval for 6 days, 3 chicks of the tumor-bearing, virus-treated group were bled and an equal amount of blood from each was pooled. At the same time the tumors were removed and pooled. A suspension of each pool was titrated intracerebrally in mice to determine the LD_{50} titer of the RSS virus present in the blood and in the tumor tissue. Blood from a group of 20 nontumor-bearing birds inoculated with RSS virus was treated in similar manner. From the results shown in Fig. 1, it is apparent that the virus concentration was the highest, and persisted the longest, in the tumor tissue.

Experiments with West Nile, St. Louis, Japanese B encephalitis and louping-ill viruses. Most of the chicks survived inoculation of tumor in one side of the breast when they were treated in the same side 3 to 4 days later with any one of the above 4 viruses (Table III). While the relative oncolytic effectiveness of these agents remains to be determined, the impression gained was that the tumors exhibited a delayed and somewhat variable response to the West Nile virus and that the response to Japanese B encephalitis virus was also delayed. The reaction to St. Louis encephalitis and louping-ill viruses was similar to the best that was obtained with RSS virus.

Effect of other viral and rickettsial agents. Of all the other viral and rickettsial agents tested in a similar manner to that described

TABLE III.
Two-week-old Chicks Treated 3 or 4 Days After Tumor Inoculation with Suspensions of Mouse Brains Infected with Viruses Other Than RSS.

Virus	Virus dose		Virus-treated	Untreated controls
	ml	%	Survival ratio	
West Nile	.5	10	13/18	0/24
	1	10	19/25	2/37
	.5	1	8/10	3/10
Japanese B	.5	1	16/20	1/18
	.5	.1	9/10	0/7
St. Louis	.5	1	19/19	1/18
	.5	.1	10/10	0/7
Louping-ill	.5	1	17/20	2/17
	.5	.1	8/10	2/10
	.5	.01	10/10	2/10

for the above viruses, none showed any consistent oncolytic action.

Immune status of surviving birds. Chicks, in which RPL-12 tumors have regressed, usually remain healthy and are immune to challenge with the homologous tumor(18). Of the survivors from the experiments described above, 185 chicks, including representative groups from all successful forms of treatment, were challenged with the homologous tumor 1 to 3 months after complete regression of all tumors. Of these birds, 55 never had presented any palpable tumors. In each experiment, normal uninoculated birds were set aside to serve as controls in the subsequent immunity-challenge tests. Tumor development seemed a better criterion for comparison of immunity than death or survival and on this basis approximately 95% of the normal control birds proved to be susceptible, while among the 185 challenged survivors only 9 (5%) developed tumors (of these 2 died). Of 18 chicks which had survived the RSS virus-tumor mixture inoculations, 4 (22%) developed tumors after the challenge and 1 died with the disease. In 13 of these 18 chicks no tumors were detected by palpation either following the original or after the challenge tumor-inoculation. Of the total 203 chicks which were challenged, 11 died or were sacrificed in a paralyzed condition. Although a similar type of paralysis occurred in at least 3 unchallenged control chicks, the data are inadequate to give an accurate estimate of the expected incidence of paralysis in chicks of this stock. While the work of Burmester, Prickett and Belding(19) suggests that neural lymphomatosis is probably not associated with the RPL-12 tumor agent, Cottral(20) after analysis of data available, formulated the working hypothesis that immunity to a tumor does not signify immunity to the etiologic agent of lymphomatosis since many of the chicks that survived the tumor transplant died of visceral lymphomatosis at a later date.

Comments. Two observations are of interest: first, that 5 different viruses which are relatively non-pathogenic for chicks possess the ability to reverse the course of tumor development which usually destroys its host, and second, that recovered birds are resistant to reinoculation with the homologous tumor. Those viruses reported to be effective in this study have certain properties in common. They are associated with systemic infections, have predilection for nervous tissues and have a fairly wide host range although they may or may not give rise to symptoms in susceptible species. Of further interest is that they fall into two groups: the RSS and louping-ill viruses are closely related as evidenced by neutralization and complement-fixation tests, and by cross-immunity tests(21); the other 3 viruses apparently contain a common minor

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antigen(22). Using the known properties, such as host range, mode of transmission, systemic infection and predilection for nervous tissue of these 5 viruses as a possible pattern of characteristics, it would seem that the eastern and western equine encephalomyelitis viruses would answer the requirements but experiments showed that these agents had no oncolytic effect on RPL-12. Any hopes engendered by the data presented in this report must be tempered by the realization that those agents which were found to be effective are impractical for use in public health or veterinary medicine. In addition, it should be emphasized that it is probably true in chicks, as has been shown for mice(23), that any one viral agent is not effective against all types of neoplasia. It is apparent, therefore, that the search for agents which may be employed with safety must be continued along broad lines and it is possible that effective viral agents may be found which have neither the antigenic nor pathogenic characteristics in common with the neurotropic group reported above.

Conclusions. 1. RPL-12, a transplantable lymphoid tumor of chickens, can be caused to regress, without any apparent damage to the host, by superimposed inoculations of Russian Spring-Summer, West Nile, Japanese B encephalitis, St. Louis encephalitis or louping-ill viruses.

2. Chickens which have been inoculated with the RPL-12 tumor followed by a superimposed infection with any of the 5 viral agents are rendered immune, as a rule, to challenge by homologous tumor even though the original inoculation with tumor may not have produced a palpable tumor.

3. The infective titer of RSS virus in infected tumor tissue is equal to that of infected mouse brain.

4. RSS virus obtained from infected mouse brain has a high oncolytic action when introduced into the tumor area but is less effective when it is introduced at a distance from the tumor.

5. RSS virus obtained from infected RPL-12 tumor has a high oncolytic action when introduced either in the tumor area or at a distance from the tumor.

The authors wish to express their appreciation to Miss Margaret Batstone for her able technical assistance during the course of these studies.

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Activity of *p*-Aminobenzaldehyde, 3-Thiosemicarbazone on Vaccinia Virus in the Chick Embryo and in the Mouse. (17652)

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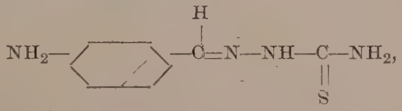
The recent addition of the thiosemicarbazones to the list of therapeutic agents for the treatment of tuberculosis(1-3) led us to investigate some of these compounds for possible activity against other pathogenic microorganisms including some of the viruses. Because it was the most soluble compound in our collection of thiosemicarbazones at the time this work was undertaken, the *p*-aminobenzaldehyde 3-thiosemicarbazone (MC2343),



was selected for first tests against vaccinia virus.

Vaccinia tests in chick embryos. In this test,

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TABLE I.
Treatment of Embryonated Eggs Infected Via the Yolk Sac with Vaccinia Virus.

			LD$_{50}$ units of vaccinia in inoculum					
			340		170		85	
Exp. No.	Treatment	Dose per egg, mg	AST*	Dead / Total	AST	Dead / Total	AST	Dead / Total
1	Saline (control)	—	96	6/6	119	5/5	109	6/6
	MC2343	0.06	128	6/6	151	6/6	191	*3*/6
	Neomycin	10	112	6/6	122	6/6	138	6/6
	γ-Globulin	18.5	121	6/6	126	6/6	139	6/6
2	Saline	—	103	12/12	104	12/12	130	12/12
	MC2343	.06	170	*10*/11	203	*8*/11	174	*5*/11
	”	.03	151	12/12	167	*11*/12	184	*6*/12
3	Saline	—	94	6/6	98	6/6	98	6/6
	10% triethylene glycol	—	90	6/6	113	6/6	113	6/6
	MC2343	.06	123	6/6	151	6/6	181	6/6
	”	.03	158	6/6	171	6/6	175	6/6
	”	.015	133	6/6	135	6/6	167	6/6
	” air sac	.05	127	6/6	160	*4*/6	147	6/6
	Myvizone (in TEG)	.01	135	6/6	114	6/6	158	6/6

* Average survival time in hours of eggs that died. Eggs surviving beyond the 19th day of incubation were not included in these averages.

6-day-old chick embryos were infected via the yolk sac with approximately 85, 170, and 340 LD$_{50}$ units of a frozen vaccinia pool (New York Board of Health strain of vaccinia). Usually 6 eggs were infected with each dose for each compound to be tested and for untreated controls. Thirty minutes after infection the eggs were treated via the yolk sac; control eggs being given saline. The eggs were incubated at 36.5°C, candled twice daily (except on Saturday and Sunday when they were candled once daily) and survival time of the eggs recorded. Chorioallantoic membranes of dead eggs were examined for lesions to establish the cause of death as due to vaccinia. Deaths of eggs prior to 70 hours after injection were usually non-specific.*

The results of 3 experiments with MC2343 are given in Table I. In the first experiment, a partially purified preparation of neomycin(4) and a γ-globulin fraction from vaccinia-immune calf serum are also included. These latter two substances both caused a significant delay in death of the eggs ($P = 0.01$), but no eggs survived beyond the 14th day of incubation; while 3 out of 6 eggs infected with 85 LD$_{50}$ units of vaccinia and treated with 0.06 mg of MC2343 survived through the 19th day of incubation. In Experiment 2, the results with MC2343 at 0.06 and 0.03 mg per egg were both promising, producing a significant delay in death of the eggs that died, and protecting 33% of those treated with 0.06 mg and 19% of those treated with 0.03 mg. In the third experiment, 0.06 mg, 0.03 mg and 0.015 mg all caused significant delays in death ($P = <0.01$) but none of the eggs survived. This variation in response to MC2343 from experiment to experiment may be due either to variation in the vaccinia content among the ampules in the frozen pool, or to variations in the response of the eggs to infection with the virus.

Since the eggs are treated by the same route as that used for infection, the possibility of a virucidal rather than a chemotherapeutic action existed. However, in another experi-

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* The tests described here are modifications of tests in eggs and in mice with vaccinia which will be reported in a publication in the near future by F. P. O. Nagler and Geoffrey Rake. The details of the present egg test, including reasons for using this experimental design and its statistical analysis (for which we are indebted to K. A. Brownlee), are also being prepared for publication.

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TABLE II.
Treatment of Mice Infected Intranasally with Vaccinia Virus.

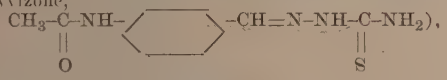
Treatment	Route	Dose, mg/kg daily	No. of mice dying day after infection 4	5	6	7	8	9	10	AST,* days	Dead Total	% surviving
Saline	SC	—		2	4	4				6.2	10/10	0
γ-Globulin	”	100			3	3	1			6.7	7/10	30
MC2343	”	40				6		2	2	8.0	10/10	0
Saline	”	—			5	4	1			6.6	10/10	0
MC2343	”	40				3	6			7.6	9/10	10
”	per os	40				1	5	1	1	8.2	8/10	20
Myvizone	”	48			1	2	7			7.6	10/10	0

* Surviving mice were omitted from calculation of average survival time.

ment, treatment with 0.06 mg delayed for 4 hours after infection gave results similar to those reported above in which treatment followed 30 minutes after infection. In addition, treatment with 0.05 mg of MC2343 via the air sac 30 minutes after infection produced a significant delay in death ($P = < 0.01$) and 2 out of the 18 eggs survived (experiment 3, Table I).

Other thiosemicarbazones are being tested against vaccinia in eggs; however, the insolubility of most of these compounds in water necessitates the use of 10-25% aqueous triethylene glycol as solvent. This solvent was chosen on the basis of its low toxicity for embryonated eggs when given via the yolk sac and its lack of significant activity against vaccinia virus in this test.

Results of one experiment with *p*-acetamido-benzaldehyde thiosemicarbazone (Myvizone,



given in Table I, experiment 3, indicate that this compound also has activity against vaccinia virus. Larger doses of Myvizone could not be tested because of its high toxicity for embryonated eggs. The approximate LD_{50} of Myvizone was 0.02 mg per egg, compared with MC2343 which was 0.125 mg per egg.

Vaccinia tests in the mouse. MC2343 was then tested in mice (12-14 g CFl strain) infected intranasally with 0.05 ml of a 10% mouse brain suspension of vaccinia. This suspension contained about 10^7 virus units per ml according to the pock count on the chorioallantoic membrane. The mice were treated with 0.5 mg (40 mg/kg) of MC2343 subcutaneously 30 minutes after infection and daily thereafter for 4 days. As a control active substance, 13 mg (100 mg/kg) of γ-globulin was given subcutaneously for the same number of doses. Control mice received saline as treatment. Results are given in Table II.

Although none of the mice treated with MC2343 survived, the delay in death of these mice was considered as confirmation of the activity of this compound against vaccinia virus in another host.

In the second experiment in Table II MC2343 was given subcutaneously to one group of mice and by mouth by catheter to another group in order to compare it with Myvizone which was given by mouth. Both drugs, when administered orally, were suspended in 5% acacia. The schedule of treatment was the same as for the first experiment.

Myvizone produced an average delay in death of the mice comparable to that produced by MC2343 subcutaneously, but none of the mice survived whereas 10% of those treated subcutaneously and 20% of those treated by mouth with MC2343 survived beyond the 25th day. These results indicate a higher activity against vaccinia virus for MC2343 than with Myvizone; however, further experiments are now in progress and a final conclusion on rank of activity must be deferred. Other thiosemicarbazones are also being tested against vaccinia virus in the mouse.

MC2343 was tested and found to be inactive, at a dose of 0.06 mg per egg via the yolk sac against about 100 LD_{50} doses of the agent of meningopneumonitis and against about 100 LD_{50} doses of the vole rickettsia (Baker). At 40 mg/kg, using the same treatment schedules as for the vaccinia experiments in mice, MC2343 was inactive against influenza virus (Shope swine influenza strain V15) in mice infected intranasally.

Summary. *p*-Aminobenzaldehyde, 3-thiosemicarbazone (MC2343) caused a significant delay in death and survival of a small percentage of chick embryos and mice infected with vaccinia virus.

At the concentrations tested this compound was inactive against the agent of meningopneumonitis, the vole rickettsia (Baker) and swine influenza virus.

Preliminary results indicated that the *p*-acetamido analogue (Myvizone) was also active against vaccinia virus.

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Excretion of Poliomyelitis Virus by Monkeys Used for Testing Material of Human Origin.* (17653)

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The variables which determine excretion of poliomyelitis virus in the stools of infected animals cannot as yet be clearly defined.(1) It was deemed desirable, therefore, to collect additional data on the excretion of virus by monkeys which develop paralysis following inoculation with material of human origin.

Methods. As part of another investigation,(2) there were available rhesus and cynomolgus monkeys which had developed paralysis following inoculation with material from a considerable number of individual patients with poliomyelitis. Each monkey was inoculated intracerebrally with the extract of throat swabs, intraabdominally with ether-treated stool extract, and intranasally with untreated stool suspension of the same patient. During the course of the nasal instillations, it was obvious that most of the 1 ml of stool suspension, which was put into each nostril each day, was being swallowed. The presence of typical poliomyelitis lesions in the olfactory bulbs of the monkeys which became paralyzed indicated that the stool suspensions which were being instilled probably contained active virus. Such animals could, therefore, be regarded as having received active virus by the oral route, the nasal route, the intraabdominal route and possibly, also, the intracerebral route. The monkeys which received the human material were killed by chloroform anesthesia on the first day of paralysis. The colon was excised aseptically and its entire contents were removed without disturbing the mucosa and stored in the frozen state in a dry ice box. In only one instance were the contents from 2 monkeys, which received material from the same patient, pooled for testing. Otherwise, the colon contents from each monkey were tested individually. After thawing the frozen material at 37°C, it was ground in a mortar with sufficient distilled water to make approximately a 10% suspension. This suspension was allowed to sediment in long tubes for 1 hour in the refrigerator; the sediment was retained for subsequent nasal instillation in the recipient monkeys, while the supernatant liquid was treated with anesthetic ether, which was added in sufficient quantity to yield a final concentration of 20% by volume. After

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* Aided by a grant from The National Foundation for Infantile Paralysis, Inc.

[†] Senior Fellow in Pediatrics, National Research Council.

1. Sabin, A. B., *Ann. Int. Med.*, 1949, v30, 40.

2. Steigman, A. J., and Sabin, A. B., *J. Exp. Med.*, 1949, v90, 349.

TABLE I.

Test for Virus in Colon Contents of Monkeys on First Day of Paralysis Following Intracerebral, Intraperitoneal, and Intranasal Inoculation with Material from Patients with Poliomyelitis.

Data on paralyzed monkeys whose colon contents were tested							
Species	Interval between last nasal instillation and onset of paralysis, days	Monkey No.	Strain	No. of days nasal instillation given	Amt I.P. ml	Polio lesions in olfactory bulbs	Result of test for virus in colon contents*
M. mulatta (rhesus)	3	2	FRO	6	55	+	6; 0; (2)
		13-41					
		14	RIS.	6	80	n. e.	0; 0
	4	62	KNI.	6	40	+	9; (1)
		87	SIC.	7	40	+	0; 0
		1	RIC.	6	57	n. e.	0; (14)
		13-88	RIS.	6	80	n. e.	0; (16)
	5	37	HOF.	6	60	+	0; 0
		89	OBE.	6	25	+	0; (8)
	6	16-66	MOH.	6	40	0 (+)†	0; 0
	7	14-76	YEA.	10	0	+	0; 0
		36	HOF.	6	60	+	0; (9)
		11	SMI.	6	30	n. e. (+)†	0; 0
	8	34	VAU.	6	60	+	0; 0
		38	FAL.	6	20	+	0; 0
		70	PET.	6	40	+	0; (16)
	10	4	HOPP.	6	72	+	0; 0
	18	8-18	SMI.	6	30	+	0; (4)
M. cynomolgus	1	82	WAL.	6	20	+	7; 7
	2	60	FAL.	6	20	+	26; 0
		71	PET.	6	40	+	16; (11)
	4	57	COL.	6	20	+	20; (11)
		88	OBE.	6	25	+	10; (5)

* Figures refer to day after inoculation paralysis first noted in individual monkeys.

0 = Neither clinical nor histological evidence of poliomyelitis; n. e. = not examined.

(1) = Encircled figure indicates day of death of monkey exhibiting neither clinical nor histological evidence of poliomyelitis.

† (+) = Evidence obtained in tests on other monkeys indicated that the stool specimens had active virus.

shaking for 10 minutes, the lightly stoppered flask was left in the refrigerator overnight during which time most of the ether evaporated. The suspensions were then submitted to 2 cycles of centrifugation, one for 15 minutes at 2,000 r.p.m. on the horizontal machine, and the supernatant liquid at 13,000 r.p.m. in an angle centrifuge for 1 hour in the cold room. After cultures on blood agar had shown it to be free of bacteria, the final supernatant liquid was inoculated into monkeys. Each specimen was tested in 2 rhesus monkeys. The untreated sediment was given intranasally, 1 ml in each nostril daily until the material was exhausted, which was usually in about 6 days. The centrifuged, etherized, supernatant liquid was injected intracerebrally in 1 ml amounts and intraperitoneally in 10 to 20 ml amounts for 1 to 2 days, depending on the amount available. Approximately 1 ml of the centrifuged, etherized suspension was saved, frozen in dry ice, and used for a second intracerebral inoculation in all monkeys which exhibited no paralysis 7 days after inoculation. Each pair of monkeys was kept in a separate cage. They were not permitted to exercise in a common run with other monkeys in order to avoid contamination with the feces of other animals. It is perhaps noteworthy that only 5 of the 45 monkeys inoculated in this manner died of toxic or septic complications resulting from the intracerebral injection of the stool extract. All monkeys which failed to show paralysis were sacrificed 28 to 35 days after inoculation and examined histologically. It may be noted that all the positive results,

TABLE II.
Summary of Data on Presence of Virus in Colon Contents on First Day of Paralysis in Monkeys Inoculated Intranasally and Intraperitoneally with Human Stools of Proved* Virus Content.

Species	Interval between last nasal instillation of human stool and onset of paralysis, days	No. of monkeys received human stool of proved virus content	No. whose colon contents yielded poliomyelitis virus
M. mulatta (rhesus)	3 and 4	4	2 (or 3)†
"	5 to 18	11	0
M. cynomolgus	1, 2, and 4	5	5

* Monkeys No. 14, 1 and 13-88, listed in Table I, are excluded because their olfactory bulbs were not examined, and there is no way of knowing whether the stool suspensions, inoculated extraneurally, or the extract of the throat swabs, inoculated intracerebrally, contained the virus.

† One of the positive tests was obtained with a pool of the colon contents of 2 rhesus monkeys, which developed paralysis at the same time after the last nasal instillation of the same human stool suspension.

indicated in Table I, are based upon the development of typical paralytic poliomyelitis, confirmed histologically, and that there were no instances of nonparalytic infection in these animals.

Results. The colon contents of 18 paralyzed rhesus monkeys and 5 paralyzed cynomolgus monkeys were tested for virus. Insofar as these animals received material from 16 different patients, they may be regarded as representing the reactions to 16 different strains of poliomyelitis virus of human origin. Poliomyelitis lesions were present in the olfactory bulbs of all but one of the 19 donor monkeys which were examined, suggesting that the stool suspensions, which were administered intranasally, most probably contained active virus. Since the incubation period in the cynomolgus monkeys was relatively short, there was none with an interval longer than 4 days after the last nasal instillation of human stools, but the colon contents of all those tested had demonstrable virus by subinoculation. It is perhaps noteworthy that when the interval between the last instillation of human stool and the onset of paralysis in the cynomolgus monkeys was only 1 day, the colon contents produced paralysis in both recipient monkeys after an incubation period of 7 days; when the interval was 2 and 4 days, poliomyelitis did not appear in all the subinoculated monkeys and the incubation periods were considerably prolonged, suggesting that smaller amounts of virus may have been present. In the rhesus monkeys, the only positive results were obtained with the colon contents of the animals in which the interval between the last nasal instillation of human stool and the onset of paralysis was 3 and 4 days. The summary of the data shown in Table II indicates that when the interval between the administration of human stool and the development of paralysis was 5 to 18 days, no virus could be found in the colon contents of any of 11 rhesus monkeys which received stools of proved virus content. It would appear, therefore, that in the rhesus monkeys the detection of virus in the colon contents depended upon the interval since the last administration of human stool rather than on the development of paralytic poliomyelitis. This suggests that the virus recovered from the colon contents of the rhesus monkeys probably represented the original human material which was swallowed rather than any that might have resulted from proliferation of the virus in the monkey. The absence of detectable amounts of virus in the colon contents of the remaining 11 rhesus monkeys indicates that the 9 different strains of virus with which they were infected did not possess the capacity to give rise to infection of the alimentary tract, despite the fact that the virus was swallowed, inoculated intra-abdominally, and obviously multiplied in the central nervous system.

Discussion. The work of Clark, Roberts and Preston(3) on the passage of the "MV" poliomyelitis virus through the intestinal tract

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3. Clark, P. F., Roberts, D. J., and Preston, W. S., Jr., *J. Prev. Med.*, 1932, v6, 47.

of rhesus monkeys indicated that virus can be recovered from the stools within 24 hours or less after administration. Their experiments did not indicate however, how long such excretion can persist. Faber, Silverberg and Dong(4) in reporting feeding experiments in cynomolgus monkeys suggested that 48 hours may be the period when "all ingested virus could reasonably be assumed to have left the alimentary tract." It would appear from the present study that the period of "passive" passage of virus through the alimentary tract can be as long as 4 days. The demonstration that the swallowing of small amounts of human stool (1 ml of 10% suspension per nostril per day) may lead to the excretion of poliomyelitis virus in detectable amounts, not only during the period of nasal instillation but also for about 4 days thereafter, is of some importance on several counts. First of all, it is evident that monkey stools might be a source of infection to the animal caretakers and laboratory workers, and it should be noted that 2 severe cases of poliomyelitis in laboratory workers, in all probability acquired in the laboratory, have been reported since 1941(5,6,7). Such excretion of virus may also be a source of infection to other animals. Howe and Bodian (8) have reported spontaneous infection in 2 chimpanzees housed in cages adjoining those of rhesus monkeys which were receiving nasal instillations of potent human stools, and recently Bodian(9) reported the appearance of paralytic poliomyelitis in an uninoculated adolescent rhesus monkey which was receiving desoxypyridoxine. In view of the results reported by Trask and Paul(10) and by Melnick(11) on the recovery of virus from the stools of various species of monkeys inoculated by extraneural, parenteral routes with strains of virus of recent human origin, it is noteworthy that in the present tests on rhesus monkeys, receiving large inocula of human stool extracts intraabdominally, there was no evidence of fecal excretion of virus.

Summary. Poliomyelitis virus was recovered from the colon contents of both rhesus and cynomolgus monkeys used in the routine testing of infectious human stools by means of nasal instillation, in addition to other routes of inoculation, when the interval between the last nasal instillation of infectious human stool and the first appearance of paralysis was 4 days or less. In none of 11 paralyzed rhesus monkeys in which this interval was from 5 to 18 days was virus found in the colon contents. The 4-day period following the last nasal instillation of infective human stools may be taken as the time required for elimination of virus swallowed by monkeys, when no local multiplication occurs. The present data indicate that monkeys, receiving human stool suspensions by the nasal or oral route, are a potential source of infection not only to their caretakers but also to other primates in the same animal quarters unless special precautions are taken to avoid fecal contamination. No evidence was obtained that poliomyelitis viruses of human origin can multiply in the alimentary tract of *rhesus* monkeys or be eliminated there by centrifugal spread from the central nervous system.

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4. Faber, H. K., Silverberg, R. J., and Dong, L., *J. Exp. Med.*, 1948, v88, 65.

5. Sabin, A. B., and Ward, R., *Science*, 1941, v94, 113.

6. Sabin, A. B., *J. A. M. A.*, 1947, v134, 749.

7. Wenner, H. A., and Paul, J. R., *Am. J. Med. Sc.*, 1947, v213, 9.

8. Howe, H. A., and Bodian, D., *J. Exp. Med.*, 1944, v80, 383.

9. Bodian, D., *Am. J. Hyg.*, 1948, v48, 87.

10. Trask, J. D., and Paul, J. R., *Ann. Int. Med.*, 1942, v17, 975.

11. Melnick, J. L., *J. Immunol.*, 1946, v53, 277.

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Effects of Liver Residue and Chorionic Gonadotropin on Ovarian Development in the Hyperthyroid Rat.* (17654)

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It has been demonstrated that massive doses of thyroid inhibit ovarian development in the immature rat, ovaries remaining infantile both in weight and microscopic appearance(1). This effect may be completely counteracted by the oral administration of desiccated whole liver(2). The protective factor in liver is retained in its water-insoluble fraction and is apparently distinct from any of the known nutrients(3,4). In the present communication data are presented indicating that chorionic gonadotropin is also effective in counteracting ovarian inhibition in the immature hyperthyroid rat.

Procedure and Results. Three experimental rations were employed in the present experiment: diets A, B and C (Table I). Diet A was a purified ration containing the B vitamins in synthetic form only. Diet B was similar in composition but contained in addition U.S.P. desiccated thyroid, added at a level of 0.5% of the ration. Diet C was similar to diet B but was supplemented with 10% liver residue powder,† added in place of an equal amount of sucrose. Previous findings indicate that this material is a potent source of one or more factors effective in counteracting the ovarian inhibition of immature rats fed massive doses of thyroid(4). Sixty female rats of the Long-Evans strain were selected at 21 to 23 days of age and an average weight of 44.2 g for the present experiment. Animals were placed in metal cages with raised screen bottoms to prevent access to feces and were fed *ad lib* the diets listed above. Ten rats each were fed diets A and C; the remaining 40 animals were placed on diet B.

Thirty-one of the 40 rats on diet B survived the 36th day of feeding. These animals were then divided into 2 groups consisting of 12 rats each. Seven animals were discarded due to infection, poor condition or atypical growth. Each rat in Group I received 80 International Units of chorionic gonadotropin‡ over a 72 hour period, consisting of 4 daily intraperitoneal injections of 20 International

TABLE I.
Composition of Experimental Diets.

Dietary component	Diet A	Diet B	Diet C
Thyroid*		0.5	0.5
Liver residue powder†			10.0
Casein‡	22.0	22.0	22.0
Salt mixture§	4.5	4.5	4.5
Sucrose	73.5	73.0	63.0

To each kg of the above diets were added the following synthetic vitamins: thiamine hydrochloride, 72 mg; riboflavin, 9 mg; pyridoxine hydrochloride, 15 mg; calcium pantothenate, 67.2 mg; nicotinic acid, 60 mg; 2-methyl-naphthoquinone, 5 mg; and choline chloride, 1.2 g.‖ Each rat also received 3 times weekly the following supplement: cottonseed oil (Wesson) 500 mg, alpha-tocopherol acetate 1.5 mg and a vitamin A-D concentrate containing 50 U.S.P. units of vitamin A and 5 U.S.P. units of vitamin D.¶

* Thyroid Powder, U.S.P., Armour and Co., Chicago, Ill.

† Extracted Liver Residue, Wilson Laboratories, Chicago, Ill.

‡ Vit. Test Casein, General Biochemicals, Inc., Chagrin Falls, Ohio.

§ Sure's Salt Mixture No. 1(5).

‖ In view of the increased requirements for thiamine, pyridoxine, and pantothenic acid in the hyperthyroid rat(6), the B vitamins in the present experiment were administered in excessive amounts in order to assure an adequacy of these factors in the diet.

¶ Nopco Fish Oil Concentrate, assaying 800,000 U.S.P. units of vitamin A and 80,000 U.S.P. units of vitamin D per gram.

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* Communication No. 247 from the Department of Biochemistry and Nutrition, University of Southern California.

1. Ershoff, B. H., *Endocrinology*, 1945, v37, 218.
2. Ershoff, B. H., PROC. SOC. EXP. BIOL. AND MED., 1947, v64, 500.
3. Ershoff, B. H., *Arch. Biochem.*, 1947, v15, 365.
4. Ershoff, B. H., *J. Nutrition*, 1949, v39, 259.
5. Sure, B., *J. Nutrition*, 1941, v22, 499.
6. Drill, V. A., and Overman, R., *Am. J. Physiol.*, 1942, v135, 474.

† This liver fraction consists of the coagulated, water-insoluble material remaining after removal of the extractable water-soluble substances.

TABLE II.
Effects of Liver Residue and Chorionic Gonadotropin on the Ovarian Weight of Immature Rats Fed Massive Doses of Thyroid.

Dietary group	No. of animals	Final body wt,* g	Ovarian wt, * mg
A	10	157.8 ± 6.9	45.6 ± 3.9
B—Saline series	12	118.6 ± 5.1	23.2 ± 2.1
B—Gonadotropic series	12	117.8 ± 4.0	53.8 ± 4.4
C	10	173.2 ± 5.9	49.7 ± 5.2

* Including standard error of the mean calculated as follows: $\sqrt{\frac{\epsilon d^2}{n}} / \sqrt{n}$ where ''d'' is the deviation from the mean and ''n'' is the number of observations.

Units each. Group II was similarly treated except that saline solution was employed. Both groups were continued on diet B for the duration of the experiment. All animals were autopsied after the 40th day of feeding (96 hours after the 1st injection); ovaries were weighed, fixed in 10% formol, and sections prepared and stained with hematoxylin and eosin.

In agreement with earlier findings a marked retardation in growth was observed in all thyroid-fed rats on the synthetic dietary regime (diet B). This effect was completely counteracted by the administration of liver residue powder (diet C). A similar situation was observed in respect to ovarian weight (Table II). Ovaries appeared immature both in weight and microscopic appearance in 11 of the 12 rats receiving saline (diet B). With one exception not a single rat in this group showed mature follicles or corpora lutea. In contrast to the above mature follicles and corpora lutea were present in 9 of the 10 rats fed diet C and 8 of the 10 animals on diet A. In addition, ovarian weight in these animals averaged approximately twice that of saline injected rats (diet B). Ovarian weight was similarly increased in animals receiving the chorionic gonadotropin. In addition, mature follicles and corpora lutea were present in 100% of the rats in this series.

Findings indicate that the ovarian inhibition of immature rats fed massive doses of thyroid is not due to an inability on the part of ovarian tissue to respond to gonadotropic stimulation. It would appear, therefore, that the failure of gonadal development in the immature hyperthyroid rat is due either to (1) an impaired formation or release of pituitary gonadotropins or (2) an increased ovarian threshold to gonadotropic stimulation. Since no inhibition of ovarian development was observed in thyroid-fed rats receiving liver residue, and since this liver fraction did not counteract other manifestations of hyperthyroidism such as an increased B.M.R., a reduction in ventricular creatine concentration, or an increase in ventricular, kidney and adrenal weight(3,4), it would seem that liver residue contains one or more factors which either (1) promote the formation or release of pituitary gonadotropins or (2) lower the ovarian threshold to gonadotropic stimulation. A third possibility is that liver residue contains an orally active gonadotropin. No data are available to indicate which of these alternatives, if any, is correct.

Summary. Ovarian development was inhibited in young rats fed massive doses of desiccated thyroid, ovaries remaining immature both in weight and microscopic appearance. This effect was completely counteracted by the administration of either liver residue or chorionic gonadotropin.

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‡ Chorionic Gonadotropin (Lyophilized), Armour and Co., Chicago, Ill. This material was dissolved in saline solution and diluted to a concentration of 20 International Units per cc.

Effectiveness of Estrogens in Stimulating Duct Growth of the Mammary Glands of Rats.* (17655)

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With the increasing number of available estrogens, we became interested in comparing their effectiveness in stimulating duct growth of the mammary glands. Although the male mouse is admirably suited for such work [Lewis and Turner(1)], the weanling male rat is not, since its mammary gland shows enlarged end buds which characterize a gland that is increasing in area. The prolonged injection of an estrogen into such a rat will increase gland area, but shorter periods of injections produce changes that are difficult to distinguish from those occurring in the noninjected rat. Myers' studies(2) on the effect of inanition on the mammary gland of the rat, from birth to 10 weeks of age, suggested a possible means of comparing the mammary-growth-stimulating properties of estrogens. These studies showed that severe inanition retarded growth of the milk ducts during the first week but did not completely inhibit their growth. In animals held at birth weight for a longer time, the ducts ceased to grow and remained in a condition only slightly more developed than at the time of birth.

In developing an assay method and in comparing various estrogens, 592 weanling male rats were used. Male rats, 21 days old, were fed various diets in different amounts until one was found that resulted in mammary glands devoid of enlarged end buds. An estrogen was then injected daily (in 0.05 cc oil) into rats on restricted diets for various periods to determine the shortest period in which enlarged end buds could be produced. It was found that the daily feeding of 2 g per rat of a diet consisting of 50% Purina Fox Chow and 50% corn starch resulted in mammary glands free of enlarged end buds. Eight daily injections of an estrogen were found to be the minimum number that would consistently produce enlarged end buds of the mammary gland. In all subsequent work 21-day-old male rats were fed the restricted diet for 10 days and injected with an estrogen during the last 8 days of the feeding period. The rats were killed on the day after the last injection. The right abdominal mammary gland of each was removed, fixed, stained *in toto* with Mayer's hemalum, and examined for evidence of duct growth. After the initial assay, for any given estrogen, the dosage was either reduced one-half or doubled until a level was attained that produced a 50 ± 10% response in a group of 10 rats.

In 409 rats that were used to compare various estrogens, the average initial body weight was 39 g; on the first day of injection it was 34 g; and at the time of autopsy it was 31 g. Of the 9 estrogens assayed, diethylstilbestrol and hexestrol showed the greatest activity whereas their dimethyl ethers showed the least. The monomethyl ether of diethylstilbestrol was more effective than the dimethyl ether but much less active than diethylstilbestrol. Estradiol and estradiol benzoate ex-

TABLE I.
A Comparison of the Mammary Stimulating Properties of Nine Estrogens.

Estrogen	Effective daily dose in μg
Estradiol	.06
” benzoate	.06
” dipropionate	.13
Estrone	2
Diethylstilbestrol	.03
Monomethyl ether of diethylstilbestrol	1
Dimethyl ether of diethylstilbestrol	8
Hexestrol	.03
Dimethyl ether of hexestrol	8

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* Paper of the Journal Series, New Jersey Agricultural Experiment Station, Rutgers University, the State University of New Jersey, Department of Dairy Industry.

1. Lewis, A. A., and Turner, C. W., *Mo. Agr. Exp. Sta. Res. Bull.*, 1939, 310.

2. Myers, J. A., *Am. J. Dis. Child.*, 1919, v17, 311.

hibited similar activity but the dipropionate was only about one-half as effective. Estrone was found to be much less effective than estradiol. The effective daily dose of 9 estrogens is given in Table I.

It will be noted that the estrogens differ greatly in their ability to stimulate duct growth. Whether or not the restricted feeding regime decreases the mammary gland response to estrogen cannot be determined in the growing male rat. Trentin and Turner(3) however, have shown that as the food intake decreases, the amount of estradiol benzoate required to produce a minimum duct growth response of the mammary glands of male albino mice is considerably and proportionately increased. Our results contrast markedly with those of Astwood *et al.*, who reported that the daily injection of 5 μg of estrone for 14 days into young rats on a restricted diet caused no duct extension whatsoever.

Summary. A method was developed whereby it is possible to use the immature male rat to compare estrogens for their ability to stimulate mammary duct growth. Nine estrogens were compared and, in increasing order of effectiveness, they were: dimethyl ether of diethylstilbestrol and dimethyl ether of hexestrol; estrone; monomethyl ether of diethylstilbestrol; estradiol dipropionate; estradiol benzoate and estradiol; and, diethylstilbestrol and hexestrol.

The author wishes to thank Mr. Clifton J. Yard for technical assistance. The estradiol (Ovocylin), estradiol benzoate (Ben-Ovocylin), and estradiol dipropionate (Di-Ovocylin) were generously supplied by Ciba Pharmaceutical Products, Inc., Summit, N.J.; diethylstilbestrol and hexestrol and their dimethyl ethers by White Laboratories, Inc., Newark, N.J.; estrone (Theelin) by Parke, Davis and Co., Detroit, Mich.; and monomethyl ether of diethylstilbestrol (Monomestrol) by Wallace and Tiernan Products, Inc., Belleville, N.J.

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3. Trentin, J. J., and Turner, C. W., *Endocrinology*, 1941, v29, 984.

4. Astwood, E. B., Geschickter, C. F., and Rausch, E. O., *Am. J. Anat.*, 1937, v61, 373.

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Normal Discontinuity of Progression of Dental Caries.* (17656)

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When appraising the presumed efficacy of a specific agent to prevent tooth decay, it is common practice to note the length of time during which the subject remains free from caries advance, without regard for the possibility that non-advance may be encountered without the use of any imposed regimen. If tooth decay normally is an intermittent process, as determined through clinical or roentgenologic examination, then one may expect non-advance to be evident at times regardless of the agent being tested. It becomes important to set up bases for prediction as to the likelihood of such non-advance and its probable duration.

A previous publication has reported that intermittency of caries progression had been noted among children being seen recurrently as patients in a dental clinic(1). Because dental therapy in itself may be expected to modify the progression of tooth decay, it was desirable to determine the intermittency of caries advance among a population for whom dental therapy was not being provided. We have done this in a state-controlled custodial institution, using teen-aged children as sub-

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* This study was made possible through a grant from the Sugar Research Foundation.

1. Boyd, J. D., and Cheyne, V. D., *J. Pediat.*, 1946, v29, 157.

TABLE I.

Characteristics of Incidence and of Advance of Dental Caries During a Two year Period Among 212 Teen-aged Subjects Living Under Standard Conditions in a Custodial Institution.

			Duration of non-advance of tooth decay (months)									
			(a) Longest consecutive period					(b) Aggregate duration of non-advance				
				In terms of initial extent of caries:		In terms of annual caries increment rate:			In terms of initial extent of caries:		In terms of annual caries increment rate:	
	Initial caries incidence: No. of DMF surfaces*	Avg annual caries increment: new DMFTS* per annum (for %ile rating total increment)	All subjects	Lowest quartile	Highest quartile	Lowest quartile	Highest quartile	All subjects	Lowest quartile	Highest quartile	Lowest quartile	Highest quartile
Mean value	8.4	2.75	9.7	11	9	13.4	6.8	11.3	11.5	11.6	14.6	8.2
Decile and quartile distribution:												
10th	1	1	3	5	0	5	0	0	3	0	5	0
20th	3	2	5	6	5	6	5	6	6	5	6	5
25th	4	3	6	6	6	6	5	6	6	6	10	6
30th	4	4	6	6	6	7	6	6	6	6	11	6
40th	5	5	6	6	6	11	6	10	11	9	13	6
50th	7	6	7	11	6	12	6	11	11	11	17	7
60th	8	6	11	11	7	17	6	12	12	12	17	10
70th	9	7	12	12	12	18	7	15	14	14	18	11
75th	11	7	15	17	14	18	7	17	17	17	23	12
80th	11	8	17	18	17	23	11	17	18	17	23	12
90th	15	10	17	23	17	23	15	19	23	17	23	15
100th	52	26	24	23	24	24	18	24	23	24	24	18

* DMF implies decayed, missing or filled; TS implies tooth surfaces

jects. Minimum amounts of dental care had been supplied to these subjects in the past, and only emergency treatment was given during the course of this study. During the period of study relating to this report, no regimentation or alteration of standard conditions of institutional life was employed.

Two hundred twelve teen-aged children (102 boys, 110 girls) were given serial dental examinations from July, 1946 to July, 1948, with at least 23 months lapse between initial and final examination for each subject. Painstaking clinical examinations were recorded and supplemented with roentgenograms of the teeth. The data were tabulated to indicate the number of newly affected surfaces of teeth in each mouth for each interval between examinations, as well as the duration of periods of non-detectable advance of caries. The findings are summarized in Table I.

Advance of caries occurred among most of the subjects during the 2-year period of study: the mean annual increment of newly affected tooth surfaces was 2.75. In 90% of the subjects, periods of non-advance were interspersed among periods of advancing caries. One-third of the group had consecutive periods of at least one year during which caries did not advance in detectable degree; in more than 80% of the group there were consecutive periods of 6 months or longer without advance. In 60% of the group, no advance was noted for an aggregate of 8 months or longer during the 2-year observation period. When the experimental group was divided into quartiles in terms of the relative net rate of progression of tooth decay, it was found as one would predict, that the quartile with the least progression of caries had had the highest mean consecutive and aggregate duration of non-advance of caries. But among the quartile with the greatest advance of caries, the median aggregate duration of non-advance was 7 months; the middle one-half of values for that quartile ranged from 6 to 12 months. Comparison also has been made between the duration of non-advance and the number of tooth surfaces affected by tooth decay at the initial observation. No difference was discernible between the quartile with the least initial extent of decay and the quartile with the most; the median duration of non-advance was the same for the two contrasted quartiles: 11 months. Likewise the range of values for the middle one-half of the subjects from each quartile was the same: from 6 to 17 months.

Thus one would have been misled if he had attempted to correlate the incidence or duration of non-advance of tooth decay with any imposed regimen. It seems obvious that in any test study wherein conclusions are to be based on the duration of non-advance of caries, the degree of non-advance in a non-controlled population of like attributes should be determined for reference.

Conclusions. One may assume either that the progress of tooth decay is discontinuous, or that there are periods of latency prior to the time when a carious area becomes detectible either to clinical or to roentgenologic examination.

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Effect of Piperidylmethyl Benzodioxane on the Cold Pressor Response.* (17657)

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Wolf and Hardy(1) have shown that the afferent limb of the cold pressor response is mediated through nervous pathways. Reiser and Ferris(2) studied the effect of tetraethyl ammonium chloride on the cold pressor response and found that this drug eliminated the response. Since tetraethyl ammonium chloride causes a transient block of autonomic ganglia, they concluded that the rise in blood pressure on immersion of the hand in cold water was on a neurogenic basis. However, in 12 of the 20 patients under autonomic blockade with tetraethyl ammonium chloride, a delayed cold pressor response occurred after removal of the hand from cold water. They believed that the delayed response could best be explained by a humoral mechanism. Dibenamine, which is capable of adrenergic blockade, inhibited the cold pressor response in 11 of 15 patients(3). It has recently been shown by Morrison and Farrar(4) that tetraethyl ammonium bromide blocks the release of epinephrine from the adrenal medulla. Hence, it is conceivable that the action of tetraethyl ammonium chloride in eliminating the cold pressor response is by blocking the release of epinephrine. Since piperidylmethyl benzodioxane in proper dosage is said to have a purely adrenolytic action(5), it was thought worthwhile to study the effect of this compound on the cold pressor response to determine whether or not epinephrine is responsible for the rise in blood pressure on immersion of the hand in cold water.

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* Part of thesis to be submitted by B. Berris to the faculty of the Graduate School of the University of Minnesota in partial fulfillment of the requirements for the degree of Master of Science in Medicine.

1. Wolf, S., and Hardy, J. O., *J. Clin. Invest.*, 1941, v20, 521.

Method. Six subjects were studied. One was in apparent good health, one had chronic glomerulonephritis, and 4 had essential hypertension. After a stable base line blood pressure was obtained with the patient in the supine position, 4 or 5 cold pressor tests were done in the usual manner, by immersion of the hand in water at 4°C for one minute. During immersion the blood pressure was recorded at the end of ½ and one minute, and every ½ minute for the next 2 minutes. An intravenous infusion of 5% glucose in distilled water was started, and piperidylmethyl benzodioxane was injected into the tubing in the dosage of 10 mg per square meter of body surface, taking 2 minutes for the injection. This was done to determine what effect benzodioxane alone would have on the blood pressure. The blood pressure was recorded every minute following the injection for 20 minutes, at which time the blood presure had returned to baseline levels. The same dose of benzodioxane was again administered in the same manner. Cold pressor tests were then repeated 2 and 6 minutes after the completion of the second injection. The rise in systolic and diastolic blood pressure on exposure to the cold stimulus before the administration of benzodioxane was averaged for each patient. An average was similarly obtained for the cold pressor tests performed during the period of activity of benzodioxane.

Results. The piperidylmethyl benzodioxane did not block the cold pressor response in any of the 6 patients studied. Furthermore, there was no significant difference in the magnitude of the response to cold stimulus after benzodioxane administration. The result in one typical experiment is shown graphically in Fig. 1. The average rise in blood pressure on cold pressor stimulation, both before and after administration of benzodioxane, are listed for all 6 patients in Table I.

Comments. The effect of benzodioxane on the response to intravenously administered epinephrine was not carried out in these patients, since 5 of the 6 had hypertension and it was not considered safe to do this. How-

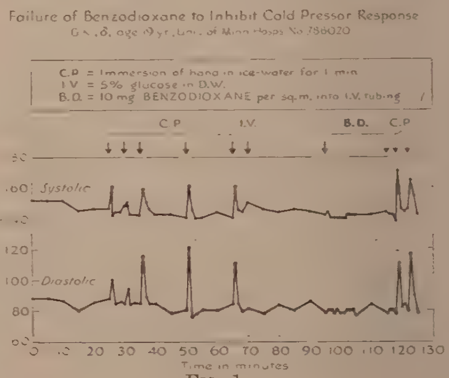

FIG. 1.

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2. Reiser, M. F., and Ferris, E. B., Jr., *J. Clin. Invest.*, 1948, v27, 156.

3. Hecht, H. H., and Anderson, R. B., *Am. J. Med.*, 1947, v3, 3.

4. Morrison, J. L., and Farrar, C. H., PROC. SOC. EXP. BIOL. AND MED., 1949, v71, 235.

5. Goldenberg, M., Snyder, C. H., and Aranow, H., Jr., *J. A. M. A.*, 1947, v135, 971.

TABLE I.

Diagnosis	Avg rise in blood pressure in cold pressor tests—MM Hg			
	Control		After benzodioxane	
	Systolic	Diastolic	Systolic	Diastolic
Normal	6	11	3	14
Chronic glomerulonephritis	14	25	23	33
Essential hypertension	19	29	30	25
,, ,,	30	25	18	14
,, ,,	21	15	22	10
,, ,,	6	13	16	16

ever, Goldenberg *et al.*(5) have demonstrated that benzodioxane abolished the rise in systolic blood pressure that followed the intravenous infusion of epinephrine. The dose of epinephrine which they used was adjusted to cause a 25% increase in systolic pressure. This increase is comparable to the increase in systolic blood pressure noted on cold stimulus in our experiments. The fact that benzodioxane did not block the cold pressor response suggests that this response is not due to the release of epinephrine. The fact that cold stimulation causes a rise in both systolic and diastolic blood pressure also suggests that this response is on a neurogenic rather than an epinephrine-induced basis.

Conclusions. Benzodioxane, administered intravenously in dosage of 10 mg per sq m of body surface, does not inhibit the cold pressor response.

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Adsorption by Erythrocytes of Antigens of *Pfeifferella mallei* and *Pfeifferella whitmori*.* (17658)

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During the course of experiments on bacterial haemagglutination it has been observed that erythrocytes of various species, under certain conditions, adsorb serologically specific substances from a solution of mallein (a Seitz E-K filtrate of a steam-killed 6 weeks culture of *Pf. mallei* in synthetic broth, concentrated to 2/5 of the original volume). Thus it was found that human, horse, guinea pig, sheep and fowl red cells exposed in contact with mallein were rendered agglutinable by the sera of horses, rabbits and other animals immunized against mallein. Ox red cells were shown to adsorb antigen from mallein solutions, and then, when treated with anti-mallein sera, to adsorb antibodies responsible for the agglutination of sensitized horse cells. However, neither ox nor pig cells exposed to mallein could be agglutinated by such antisera.

Sheep and fowl cells required about 8 times, and guinea pig cells 2 times more mallein to sensitize them than did horse and human cells. Different batches of mallein varied considerably in their sensitizing potency, some being twenty times more active than others in this respect. There was no quantitative relationship between the amount of sensitizing antigen and the amount of antigen responsible for complement fixation present in the various preparations.

Sensitization of horse red cells with a minimum sensitizing dose of antigen took 3 hours to reach completion at room temperature

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* This work has been carried out at the Institute of Animal Pathology, Cambridge, England, on a grant from the Animal Health Trust, London.

TABLE I.

Agglutination of Horse Erythrocytes Sensitized with Mallein by Normal and Immune Sera.

Serum dilutions	Horse serum		Rabbit serum		Human serum	
	Normal (1:10)	Anti-mallein (1:5120)	Anti-*Salmonella pullorum*	Anti-mallein	Normal	From patient infected with *Pf. whitmori* (640)
80					—	++++
160					—	++++
320	++++	++++	—	++++	—	++++
640	++++	++++	—	++++	—	++++
1,280	—	++++	—	++++	—	++++
2,560	—	++++	—	++++	—	++++
5,120	—	++++	—	++++	—	++++
10,240	—	++++	—	++++	—	++++
20,480	—	++++	—	+	—	—
40,960	—	++++	—	—	—	—
81,920	—	++	—	—	—	—
123,840	—	—	—	—	—	—
327,680	—	—	—	—	—	—

Note: The figures in parentheses refer to the titers of the sera by the Conglutinating Complement Adsorption Test [Hole and Coombs(2)].

(about 21°C) whereas, at 37°C the reaction was complete in 15 minutes or less.

Horse red cells were generally used in serum titration experiments for a number of reasons, *e.g.*, they were readily available in quantity, they required less antigen than other species to sensitize them, and positive sera agglutinated sensitized horse cells to a higher titre than sensitized sheep or fowl cells.

The following technique was used to titrate sera for antibody against the adsorbable antigens. Two minimum sensitizing doses of mallein (0.01 ml of a potent preparation) were added in 1 ml of saline to 1 ml of a 5% suspension of horse red cells (previously washed at least 4 times in saline); this mixture was incubated for one hour at 37°C. The cells were then washed twice and resuspended in 1 ml saline. One drop (0.05 ml) of this suspension was added to each tube (0.8x7 cm) of a row containing twofold dilutions in 0.5 ml quantities of the test serum. The tubes were then shaken and allowed to stand for one hour at room temperature. Agglutination was manifest by the formation by the cells on the bottom of the tubes of a pattern similar to that seen characteristically in haemagglutination by the influenza group of viruses [Salk(1)].

Some samples of sera, especially from the horse, contained substances inhibiting the formation of the typical agglutination pattern. This effect was observed in dilutions of serum up to 1:1280. In such cases, the agglutinated cells settled to the bottom of the tube more quickly and could be seen flocculating together in grossly visible aggregates which were readily dispersible on shaking. If the tube was allowed to stand undisturbed for some time, these floccules tended to slide down the sloping surfaces of the bottom of the tube to the lowest and central point, so that the final picture was similar to that seen in the saline controls and with negative sera. Kaolin, in the proportions of 1 g to 10 ml of serum dilution 1:10 in phosphate buffer (pH 7), was found capable of completely adsorbing the inhibitory factor, while causing only a slight drop in antibody titre.

Some typical results are shown in Table I.

The agglutination of mallein-sensitized cells by the serum of the patient suffering from infectious melioidosis is of interest. This serum has also been shown to agglutinate cells sensitized with a similar antigen preparation from *Pf. whitmori*, although this preparation failed to sensitize the cells to agglutination by anti-mallein serum except when used in much higher concentrations (100 fold). Even then,

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1. Salk, J. E., *J. Immunol.*, 1944, v49, 87.

2. Hole, N. H., and Coombs, R. R. A., *J. Hyg. Camb.*, 1947, v45, 480, 490.

the agglutination was weak and uncertain, and only occurred in sera in relatively high concentrations.

Little work has so far been carried out on the chemical nature of the adsorbable antigen and of the receptor on the red cell responsible for the adsorption. The experiments of Keogh and North(3) with *H. influenzae* and other organisms, and of Middlebrook and Dubos(4) using saline extracts of tubercle bacilli after extracting with 90% phenol, suggest that a bacterial polysaccharide, either alone, or in combination with some other substance, may be involved.

In connection with the receptor on the red cell, the following experiment may be worth reporting. An alcohol-ether (3:1) extract of 10 ml of washed packed horse red-cells was prepared: the extract was evaporated and the residue resuspended in 25 ml saline with the aid of a little ether. Two rows of tubes were set up each containing twofold dilutions in 0.5 ml of the red cell extract suspension, and were allowed to stand at 37°C to let the ether evaporate. To the tubes of the first row were added 2 sensitizing doses (for 1 drop 5% red cells) of mallein, in 0.1 ml saline; after one hour at 37°C one drop of 5% horse red cells were added, and the mixture incubated for another hour. In the second row, the red cells were added first, and the sensitizing antigen at the end of the first hour. Then all the tubes were centrifuged and the cells washed twice, before adding to each two agglutinating doses of an anti-mallein serum, contained in 0.5 ml saline. The results were as follows:

Tube No.	1	2	3	4	5	6	7	8	9	10
Row 1	—	—	—	—	—	—	2+	3+	4+	4+
Row 2	—	+	+	+	2+	4+	4+	4+	4+	4+

The alcohol-ether extract, therefore, contained some substance or substances capable of inhibiting the sensitization of horse red cells by mallein to agglutination by anti-mallein sera.

The difference between the rows 1 and 2 is consistent with the interpretation that this inhibition is due to an affinity of the inhibitor for the antigen rather than to a mechanical blocking effect resulting from a coating of the red cell surface. In other words, there appears to be true competition between the extracted substance and the red cells for the antigen.

Summary. Erythrocytes of the horse and some other species treated with mallein were rendered thereby agglutinable by high dilutions of anti-mallein sera and by the serum of a human patient suffering from infectious melidioidosis. Erythrocytes of ox and pig could not be sensitized to agglutination under the same conditions. The sensitization by mallein of horse red cells to agglutination by antimallein sera was inhibited by an alcohol-ether extract of these cells.

My thanks are due to Professor W. I. B. Beveridge, Dr. R. Scarisbrick, and Dr. H. P. Chu for much useful advice, and also to Dr. R. R. A. Coombs and Mr. N. H. Hole for kindly supplying the mallein and some of the sera.

Received January 23, 1950. P.S.E.B.M., 1950, v73.

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3. Keogh, E. V., North, E. A., and Warburton, M. F., *Nature*, 1948, v161, 687.

4. Middlebrook, G., and Dubos, R. J., *J. Exp. Med.*, 1948, v88, 521.

Effect of Long-Term Panthenol Administration on the Pantothenate and Coenzyme A Contents of Rat Tissues.* (17659)

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Numerous studies(1-8) have shown that panthenol, the alcohol analog of pantothenic acid, exhibits quantitatively the known biological effects of pantothenic acid, and, moreover, has the advantage of greater stability *in vitro*(9) as well as better availability in mammals at therapeutic dosages(2-5). On the other hand, panthenol was reported by Snell and Shive(10) to have an *in vitro* anti-pantothenate effect on certain microorganisms, *e.g.*, antibacterial indices of 300, 1000 and 5000 against *Leuconostoc mesenteroides*, *Streptococcus lactis* 125, and *Lactobacillus arabinosus*, respectively. Panthenol had no effect, however, against microorganisms such as *Escherichia coli* and *Staphylococcus aureus*, which are capable of synthesizing pantothenic acid. In all cases where panthenol competitively inhibited the microbiological growth effects of pantothenic acid, the inhibition was overcome by the presence of sufficient pantothenate. It is clear therefore that this anti-pantothenate effect stems from the circumstance that these microorganisms are incapable of oxidizing panthenol to the acid, a function which mammals perform efficiently.

The present experiments were designed to test whether any anti-pantothenate effect might be detected when high doses of panthenol are administered to rats, mice, dogs and rabbits receiving a stock diet adequate with regard to pantothenic acid content. As a measure of possible anti-pantothenate action, consideration has been given to (1) acute toxicity, (2) chronic toxicity, as evidenced by the production of histological or hematological changes and alteration of the normal rate of growth, and (3) influence of long term feeding upon tissue stores of pantothenic acid. In regard to the last Novelli *et al.*(11) have established that practically all the pantothenic acid found in mammalian tissues may be accounted for as the coenzyme of acetylation, coenzyme A. For this reason, both coenzyme A and free pantothenate as well as total tissue stores were determined in testing for panthenol-induced changes in the storage of pantothenic acid by the rat.

Experimental. (1) *Acute Toxicity.* In acute toxicity studies of panthenol, the intravenous LD_{50} for mice was 7 g per kilo and for rabbits 4 g per kilo. All the rabbits survived 3 g per kilo intravenously and the mice 6.25 g per kilo.

(2) *Chronic Toxicity.* The rate of growth of 24 rats receiving 2 mg of panthenol daily for 6 months was compared to that of 12 control rats receiving the same stock diet, which supplied an adequate amount of pantothenic acid.† As the curves (Fig. 1) relating weight gains to duration of supplementation were practically superimposable over the entire period, it is evident that long term administration of panthenol to growing rats had no

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* Presented in part at the 116th Meeting of the American Chemical Society, Abstracts p. 65C, September 23, 1949.

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10. Snell, E. E., and Shive, W., *J. Biol. Chem.*, 1945, v158, 551.
11. Novelli, G. D., Kaplan, M. O., and Lipmann, F., *J. Biol. Chem.*, 1949, v177, 97.

† This dog chow contains 10 μg of pantothenic acid per gram, *i.e.*, sufficient to provide a daily intake of 100 to 150 μg per rat per day.

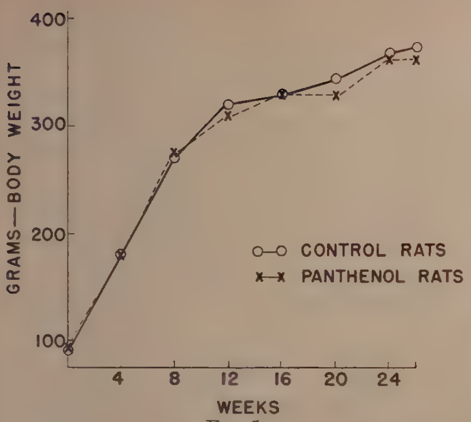

FIG. 1.

Effect of feeding 2 mg of panthenol per day upon the rate of growth of normal rats.

deleterious effect. In a similar study lasting 3 months, 20 mg of panthenol fed daily to rats and 500 mg to dogs produced no histopathological changes. White and red blood cell counts, hemoglobin determinations, nonprotein nitrogen and blood sugar levels showed no deviation from normal.

(3) *Influence of Long Term Feeding upon Tissue Stores of Pantothenic Acid.* Daily doses of 2 mg of panthenol or an equivalent amount (2.3 mg) of calcium pantothenate were fed to rats maintained on the above stock ration for test periods of 24 and 45 days and for 5 to 6 months. Undosed control rats were given the same diet. In the case of the 24 and 45 day test animals, the panthenol or calcium pantothenate was administered by stomach tube and all groups were parallel. For the 5 to 6 months tests, the rats received the same supplements as a part of their ration. Although in these longer experiments, the 5-month test with the calcium pantothenate group was not run simultaneously with the 6-month test of panthenol and control groups, conditions were otherwise controlled so that all three groups are comparable.

(a) *Total pantothenate.* The total pantothenate content of liver, heart, kidney, and spleen tissues from these rats was estimated (Table I) after complete liberation of the pantothenic acid from its naturally occurring bound form as coenzyme A by means of the liver enzyme, alkaline phosphatase procedure described by Novelli *et al.*(11). The liberated vitamin was assayed microbiologically with *L. arabinosus (17-5)* by the method of Skeggs and Wright(12). In the early experiments, the assays were read turbidimetrically with color blanks, while in the later tests, titrimetric measurements were employed. Assays of the same samples by both procedures showed close agreement.

Pooled livers from each of the 3 groups in the 24- and 45-day tests were analyzed simultaneously as soon as the rats were sacrificed. It is evident (Table I) that in these tests neither panthenol nor pantothenate administration appreciably affects the total pantothenic acid content of rat livers. Similarly, the data obtained after 5 to 6 months show no significant increase of liver stores in the dosed rats. Parallel analyses of pools of kidneys from the control, panthenol and pantothenate groups after 24 and 45 days and hearts from the 5 to 6 months series indicate only slightly increased stores of pantothenate after both substances. In the 5 to 6 months series, the 42% increase in kidney content after panthenol compared to only 2% after calcium pantothenate cannot be explained at this time. In contrast, splenic storage was unaffected under these conditions.

(b) *Test for unconverted panthenol.* It was also of interest to determine whether panthenol *per se* is stored in the tissues of these rats. To test this possibility, the microbiological assay procedure of De Ritter and Rubin(13), using *Acetobacter suboxydans* (A.T.C.C. 621 H), was applied to an enzyme-treated suspension of 3 livers from the panthenol-dosed rats of the 6 months test. Following complete alkaline hydrolysis of an aliquot of this suspension, the pantoic acid found was equivalent to 90 γ of pantothenic acid per gram of liver. Direct determination of pantothenic acid before hydrolysis on the same day and with the same organism indicated a content of 79 γ/g. The pantothenic acid content by *L. arabinosus* assay was 93 γ/g. From the agreement of those 3 values it is evident that, under these

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12. Skeggs, H. R., and Wright, L. D., *J. Biol. Chem.*, 1944, v156, 21.

13. De Ritter, E., and Rubin, S. H., *Anal. Chem.*, 1949, v21, 823.

conditions, no significant amounts of panthenol or pantoic acid are stored in the liver.

(c) *Relative coenzyme A content of livers.* To test whether panthenol or pantothenate administration affects the normal coenzyme A content of liver tissue, organs from each of the various groups in the 24 and 45 tests were pooled and extracts prepared according to the procedure described by Kaplan and Lipman(14). Such extracts were reported to contain intact the coenzyme A, the integrity of which these authors proved by its activity in the enzymatic acetylation of sulfanilamide. The activity of our extracts was determined with *Acetobacter suboxydans,* which we find in agreement with Novelli *et al.*(15), utilizes the intact coenzyme. The response of this organism, grown on the medium of De Ritter and Rubin(13), was measured with reference to a pantothenic acid standard to provide an estimate of relative rather than absolute coenzyme A potencies of the livers from the various groups. A summary of the comparative data is given in Table II, all potencies being expressed in terms of "apparent pantothenate." In both the 24 and 45 day tests, panthenol and pantothenate cause a slight and equal increase in the relative activity of about 20% over the controls. In the longer test period of 5 to 6 months, neither substance affects the activity.

To demonstrate that this activity is due primarily to coenzyme A rather than to free pantothenate, which also has activity in this assay, it was necessary to determine the amount of free pantothenate present in each case.

(d) *Free pantothenate in livers.* Novelli *et al.*(11) have found that normally no more than 3% of the total pantothenate of rat heart, liver, and kidney tissue is unbound. To compare the amounts of free and bound pantothenic acid in livers from the present control and dosed groups, the coenzyme A extracts were analyzed with *L. arabinosus* both directly and after enzymatic liberation of the bound

TABLE I.
Rat Tissue Storage of Pantothenic Acid.

		Total pantothenic acid content (μg/g fresh tissue)							
		Control		Panthenol (2 mg/day)			Ca Pantothenate (2.3 mg/day)		
Organ	Test period	No. of organs*	Avg content	No. of organs	Avg content	% incr.	No. of organs	Avg content	% incr.
Liver	24 da.	2 (1)	67	2 (1)	68	2	2 (1)	67	0
	45 ,,	3 (1)	74	3 (1)	83	12	2 (1)	79	7
	5-6 mo.	7 (5)	100	9 (6)	94	—6	5 (3)	75	—25
Kidney	24 da.	10 (2)	51	10 (2)	55	9	10 (2)	57	13
	45 ,,	10 (2)	48	10 (2)	61	27	10 (2)	54	12
	5-6 mo.	12 (4)	60	16 (4)	85	42	8 (2)	61	2
Heart	5-6 ,,	10 (2)	47	14 (2)	56	20	12 (2)	62	32
Spleen	5-6 ,,	12 (2)	27	14 (2)	27	0	14 (2)	27	0

* The numbers in parentheses indicate the number of analyses performed on separate pools of organs.

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15. Novelli, G. D., Flynn, R. M., and Lipmann, F., *J. Biol. Chem.*, 1949, v177, 493.

TABLE II.
Coenzyme A Activity of Liver.

	Average Coenzyme A activity* (µg apparent pantothenic acid/g of fresh tissue)		
Test period	Control	Panthenol (2 mg/day)	Ca pantothenate (2.3 mg/day)
24 days	134	165	162
45 ”	161	190	190
5-6 mo.	199	193	183

* Determined with *Acetobacter suboxydans.*

TABLE III.
Free Pantothenate in Coenzyme A Extracts of Rat Liver.

	Pantothenic acid content* (µg/g fresh tissue)								
	Control			Panthenol (2 mg/day)			Ca pantothenate (2.3 mg/day)		
Test period, days	Free	Bound	% Free	Free	Bound	% Free	Free	Bound	% Free
24	1.4	40	3.5	2.8	67	4.2	1.7	41	4.2
45	1.6	50	3.2	2.5	55	4.5	2.8	55	5.1

* Assay with *L. arabinosus* to measure free and total pantothenate, respectively, *before* and *after* enzymatic liberation; difference equals bound pantothenate.

vitamin. As this organism is insensitive to the intact coenzyme but responds quantitatively to the free vitamin, the concentration of free pantothenic acid in the extracts is measured by the direct assays. It is evident (Table III) that practically all of the pantothenic acid present in livers of rats chronically administered either panthenol or calcium pantothenate is also in the bound form.

Discussion. In view of the anti-pantothenate activity exhibited by panthenol towards certain microorganisms(10), the present experiments have tested for such effects in mammals. Previously reported panthenol availability studies(1,2,4) have demonstrated that the rat readily converts panthenol to pantothenic acid as shown by the urinary excretion of pantothenate. As a consequence, for panthenol to exert an *in vivo* anti-pantothenate effect, it must do so before its conversion by displacing and thereby decreasing normal tissue stores of pantothenate. Comparison of the effect of chronic administration of panthenol or pantothenate upon total tissue stores have tested this possibility. In no case do we find any appreciable decrease attributable to panthenol. In fact, the total kidney and heart stores are increased slightly by both panthenol and pantothenate, whereas splenic and liver stores remain unaffected. On this basis, it is concluded that panthenol does not displace normal tissue stores of pantothenic acid.

In determining the mode of storage of pantothenic acid, the microbiological procedures employed yield only a relative estimate of the coenzyme A content of the livers from the various groups. However, these values (Table II) in terms of apparent pantothenic acid activity per gram of tissue are in direct proportion to the metabolically active coenzyme A present in the extracts(15). Preparation of our extracts by procedures reported by Kaplan and Lipmann(14) to protect the integrity of the coenzyme A molecule and to preserve its acetylation activity precludes the presence of degradation products of coenzyme A such as the pantothenic acid conjugase, reported by King *et al.*(16) to support the growth of *Acetobacter suboxydans.* The small percentages of free pantothenate and the lack of significant amounts of pantoic acid indicate that the *Acetobacter suboxydans* assay measured chiefly coenzyme A.

It is clear that panthenol and pantothenate have practically identical effects upon the

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16. King, T. E., Locher, L. M., and Cheldelin, V. H., *Arch. Biochem.*, 1948, v17, 483.

levels of both pantothenate and coenzyme A in rat tissues. There was no appreciable accumulation of free pantothenate or panthenol during these tests. The lack of inhibitory effect of panthenol upon the rate of growth, the histopathological and hematological picture, as well as the high intravenous LD_{50} found for mice and rabbits provide further evidence that panthenol has no *in vivo* antipantothenate effect. Furthermore, in clinical tests of panthenol(6,7) no untoward or toxic effects were observed. For these reasons, it must be concluded that panthenol is generally non-toxic to mammals at dosage levels in the therapeutic range.

Summary. Toxicity tests of panthenol show that this biologically active, hydroxy analogue of pantothenic acid has no *in vivo* antivitamin effect in mammals. In acute toxicity tests, mice tolerated up to 6.25 g per kilo and rabbits up to 3 g per kilo. In chronic feeding tests, 2 mg per day of panthenol had no inhibitory effect upon the growth rate of normal rats on an adequate stock diet and 20 or 500 mg daily produced no histopathological or hematological changes in rats or dogs, respectively. When fed as above at the 2 mg per day level for periods up to 6 months, panthenol did not differ significantly from an equivalent supplement of calcium pantothenate in its effect on rat tissue storage of pantothenate. Total pantothenate in the liver, spleen, kidney and heart, and coenzyme A in the liver were equal or slightly greater in the dosed groups than in the controls. Liver storage in all groups was almost entirely as coenzyme A, and no appreciable amounts of panthenol or free pantothenate accumulated.

We wish to thank Mrs. Irma Sonnenfeld and Mr. Jacob Scheiner for assistance with the tissue studies, and Miss Margaret D. Roe and Mr. Carmen Mangieri for carrying out many of the toxicity tests.

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Effect of Testosterone Propionate on Permanent Canine Tooth Eruption in the Monkey (*Macaca mulatta*). (17660)

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An obvious dimorphic character in the common rhesus monkey is the tusklike permanent canine tooth of the male. In the female the degree of modification of the canine is very much like that in human dentition. In fact, the teeth of the *Macaca mulatta* show many similarities to the teeth of man(1) and the dental formulae for both the deciduous and permanent teeth of man and this monkey are the same. In the normal male monkey of the rhesus colony† where these observations were made, the permanent canines begin to erupt during the latter half of the fourth year and all of them are present by the end of the fifth year. When recording the appearance of the deciduous teeth, it is necessary to make daily examinations during the first 3 months of infancy and then weekly observations are made, but after the second year the monkey's permanent teeth are not checked against his chart more often than once a month. There is, therefore, a plus or minus error of around 30 days in the data given here. However, in the normal male monkey all the permanent canines do not appear simultaneously. They emerge one or two at a time as in the human, their period of eruption extends over 2 to 7 months.

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* Supported by a grant from the Nutrition Foundation, Inc.

1. Marshall, J. A., *The Anatomy of the Rhesus Monkey*, edit. by Carl G. Hartman and W. L. Straus, Jr., The Williams & Wilkins Company, 1933, chap. VI, p. 85.

† The Obstetric Monkey Colony, Yale School of Medicine, New Haven, established in 1931, now consists of 5 generations of this rhesus monkey.

The dental records of a series of 38 normal and 16 experimental males born in the colony have not been analyzed in full, as yet, but on the basis of preliminary surveys of the material, certain striking differences are apparent between the ages at tooth emergence in normal animals and those receiving injections of testosterone propionate.

The dentition records of 3 experimental animals in which early bone union of the skeleton and sexual precocity were induced(2) has been compared with similar data relating to the dentition of 10 normal males. The treatment with androgen began in Mm 22 at 6 months and 21 days of age; Mm 18 at 7 months, 6 days and Mm 43 at 1 year, 10 months and 2 days. Mm 22 and Mm 18 received 7.5 mg testosterone propionate[‡] per kilo body-weight per week and Mm 43 received 22.5 mg per kilo. In these 3 monkeys an acceleration of growth and differentiation resulted in skeletal maturation at 2 years, 10 months instead of around 7 years, the age at which bone union is completed in the normal male.

TABLE I.
Data on Permanent Canine Tooth Emergence for 10 Normal Male Monkeys and 3 Males Injected with Testosterone Propionate.

	Age at emergence of earliest permanent canine			Age at emergence of the fourth permanent canine		
Animal No.	yr.	mo.	day	yr.	mo.	day
A. Control Monkeys (Males):						
25	3	11	0	4	3	1
82	3	6	29	3	10	28
86	4	1	20	4	4	28
40	4	1	24	4	8	22
49	4	2	15	4	8	7
77	3	10	11	4	2	9
83	4	0	20	4	3	19
84	3	9	18	4	1	15
53	4	3	10	4	4	14
56	4	2	22	4	7	23
Age avg	4 yr.	0 mo.	7 days	4 yr.	4 mo.	11 days
B. Monkeys injected with testosterone propionate (Males):						
18	3	3	28	3	3	28
22	3	2	1	3	2	1
43	3	2	3	(2 lower canines present; autopsied)		
Avg age	3 yr.	2 mo.	21 days			

Conclusions. A study of Table I reveals that in no normal male were the 4 permanent canines recorded as having emerged more or less simultaneously. The average age of the injected animals at time of canine emergence is seen to be about a year earlier than that of the controls. In fact, the earliest permanent canines in the latter group did not appear as early as the latest ones in the experimental group. It is thus evident that when androgen is employed as a growth-stimulating agent, the development with emergence of the canine teeth is also accelerated.

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2. van Wagenen, G., *Fed. Proc.*, 1947, v6, 219.

[‡] Oreton was supplied by the Schering Corporation through the interest of Dr. Edward Henderson.

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A Test for the Antigenicity of Rabies Vaccine. (17661)

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It is our purpose to report here a test for the immunizing ability of rabies vaccine that is more logical and less time-consuming than the official Habel test(1). It consists of one intraperitoneal injection of a vaccine into guinea pigs, followed 3 weeks later by the

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1. Habel, K., *U. S. Pub. Health Rep.*, 1940, v55, 1473.

intramuscular inoculation of street virus. The minimum amount of vaccine that will protect against a given dose of street virus is determined.

Materials and methods. The guinea pigs come from a closed stock that has been maintained in this laboratory for 30 years and is practically free from disease. In an experiment using 50 or more guinea pigs no deaths from an intercurrent infection usually occur. These guinea pigs are fed a good quality of hay, oats, and cabbage during the winter, and oats and fresh alfalfa during the warmer months. Animals weighing from 250-350 g are used and the controls are kept with those receiving vaccine. In early experiments 5 guinea pigs were injected with each dilution of vaccine but at the present time 6 or more are used.

The vaccines to be tested are diluted in buffered salt solution and 1 cc is injected intraperitoneally into each animal. The dilutions commonly used are 1:50 and 1:250 of the 10% brain suspension.

The virus used for testing immunity was the brain of a dog developing rabies in Atlanta, Ga., received from Dr. Harald Johnson in 1943. A 20% suspension of this brain and also material from the first intracerebral passage through guinea pigs have been kept in sealed ampules frozen under CO_2 ice. The challenge virus is the 20% suspension 2nd guinea pig passage brain that has also been kept in sealed ampules under CO_2.

The time between the inoculation of vaccine and the challenge is approximately 3 weeks. An ampule of the 2nd guinea pig passage virus is removed from the CO_2 box, thawed, and diluted in cold sterile skim milk that has been adjusted to pH 7.3. Guinea pigs are immediately inoculated deep into the gastrocnemius muscle with 0.5 cc, using an automatic syringe* and a 2 inch, no. 22 gauge needle. The dilution used is approximately 10 times the MLD_{50} dose and is either 1:50 or 1:100. The inoculations with street virus are made in a special room, the animals are kept in an isolation unit, and persons who care for them wear heavy rubber gloves which are placed in diluted creolin after they are removed.

A small number of susceptible animals show a furious type of rabies for a short period of time and care is taken that the workers are not bitten. The great majority of the animals develop a paralysis 9 to 15 days after inoculation and death occurs from 1 to 4 days later. With a few exceptions all deaths occur within 20 days after the injection, and it has been our practice to terminate the experiments a month after the challenge injection. If this time is extended to 2 months some of the few animals that survive in the control group may develop rabies but it is probable that this is an infection from their cage mates. No new cases occur among the vaccinated animals.

An occasional animal, either vaccinated or control, will show paralysis at the usual time but will live for a much longer period and occasionally one will recover. These animals have been called positive because the symptoms have developed at the proper time. Tests for virus in their brains and salivary glands have been negative but their sera show a much higher content of neutralizing antibodies than do sera from vaccinated guinea pigs.

Results. Street virus has been used in the past for challenge but on the whole the tests have not been satisfactory because only a portion of the control animals developed rabies. In our experiments 90% of the control animals develop rabies; for example, with one 2nd passage brain that has been in use for 17 months, 145 out of 152 normal animals have been infected.

There are several factors which have to be considered that may influence the development of the disease. The inoculum is large, 0.5 cc, and it is injected with considerable force, injuring the tissues. If the inoculum is reduced to 0.1 cc and the concentration increased so that the animals receive the same amount of virus, or if the inoculum is kept at the same level but is introduced slowly, the number of takes is reduced. The effect of the milk used as a diluent has not been determined, but it is not essential, for 10% horse serum can be used in its place. The

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* Cornwall syringe No. 1250, Becton, Dickinson and Co., Rutherford, N.J.

TABLE I. A Typical Protocol.

	10/8. Intraperitoneal injection, 1 ml		Guinea pig			10/31. Intramuscular injection 0.5 ml, 2nd guinea pig passage street virus		
						Date of		
Vaccine	Dilution	% brain	No.	Wt 10/8	Wt 10/30	Symptoms	Death	Dead/inoculated
A. 10% rabbit brain inactivated	1:1250	0.008	1	325	440			2/5
			2	350	415	11/11	11/15	
			3	340	440		11/11	
			4	280	370			
			5	350	470			
	1:250	0.04	6	290	410			0/5
			7	335	445			
			8	300	430			
			9	340	430			
			10	300	475			
	1:50	0.2	11	295	440			0/5
			12	315	440			
			13	270	420			
			14	330	430			
			15	310	430			
B. 10% guinea pig brain inactivated	1:1250	0.008	16	355	510	11/14	11/17	4/5
			17	345	460	11/11	11/16	
			18	295	425	11/11	11/13	
			19	270	395			
			20	350	460	11/ 9	11/13	
	1:250	0.04	21	325	480	11/10	11/12	3/5
			22	345	465			
			23	310	410			
			24	335	455	11/15	11/19	
			25	310	425	11/11	11/15	
	1:50	0.2	26	290	395			0/5
			27	340	475			
			28	330	425			
			29	300	425			
			30	275	405			
Controls			31	335	490	11/11	11/14	5/5
			32	315	470	11/10	11/14	
			33	325	480	11/11	11/18	
			34	265	390	11/11	11/13	
			35	270	415	11/13	11/15	

Experiment terminated 12/1.

TABLE II.
Summary of Tests on Phenol Vaccines for Canine Immunization.
Intraperitoneal injection of vaccine 8/15. Intramuscular injection of street virus 9/13.

		% brain suspension and results		
Vaccine	Days to expiration	0.5	0.1	0.02
1	294	0/7	4/5	4/5
2	54	1/7	2/5	4/5
3	8	4/7	4/5	5/5
4	215	0/7	2/5	4/5
5	49	2/7	3/5	5/5
Rabbit brain, 3 mo. after preparation		0/6	1/5	1/5
Controls			7/7	

Exp. terminated 10/14.

strain of guinea pig may be important. In one test in another laboratory on a group of guinea pigs that were in poor condition and were dying from a respiratory disease, the controls did not show a high number of takes. However, groups of our guinea pigs on a poor diet or in an environment above (80-83°F) or below (60°F) the usual room temperature (70°F) have shown the same susceptibility as control animals.

After a great number of tests of various kinds we have come to consider a vaccine satisfactory when 1 cc of a 0.2% brain suspension protects all animals and the same amount of a 0.04% protects at least half of the animals. In some vaccines we consider especially good, 1 cc of an 0.008% suspension protected. In Table I are given in detail the results of the test of a good and an excellent vaccine. In Table II are results of a test made on 5 samples, purchased on the open market, of phenol-inactivated rabies virus for the immunization of dogs, together with the vaccine prepared in this laboratory as a control. Further results on the comparison of vaccines will be given in another paper.

Discussion and summary. This is a test of the ability of a vaccine to produce immunity to street virus injected peripherally. It seems more logical than the official Habel(1) test which tests the ability of a vaccine to produce a resistant state, which may be interference or immunity, to a given strain of fixed virus injected intracerebrally. In the Habel test a given strain of fixed virus must be used, as the resistant states cannot be demonstrated to all strains of fixed virus(2,3). While most of our tests have been with a single strain of street virus, we have used enough other strains to know that they will give good results. Our work has been done with a given strain of guinea pigs, but it is probable that animals from any healthy stock can be used; whereas in the Habel test special strains of mice and animals of a given weight must be used.

The time-saving factor may not be important when a single vaccine is tested, but when many are under study it is of considerable importance. For instance, if the Habel test were used to compare the vaccines given in Table II, 300 intraperitoneal injections would be required on each of 6 days, plus 330 intracerebral injections. The time taken for inoculation of the guinea pigs in the test was not more than 2 hours. It is very desirable that comparative tests be made using the one described and the Habel test on a variety of vaccines. We have not done this because of the work involved and because the test as we have used it gave satisfactory results in comparing vaccines. In our work we considered each experiment as an entity and were not interested in securing a highly standardized test. Repeated tests made at different times on the same vaccine have given essentially the same results.

Working with street virus involves a certain

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2. Habel, K., and Wright, J. T., *U. S. Pub. Health Rep.*, 1948, v63, 44.

3. Wright, J. T., and Habel, K., *J. Immunol.*, 1948, v60, 503.

risk and this has to be considered. Carefully trained and dependable help must be used, and with such help the risk does not appear to be very great. We have not felt that vaccination of the personnel was necessary.

Some strains of fixed virus injected intramuscularly will cause death in guinea pigs in as high dilutions as the street virus and it is possible that they could be substituted for the latter with satisfactory results.

Maximum immunity develops at 3 weeks, but if more vaccine is injected the interval between the injection of vaccine and of virus can be shortened.

Injury to the tissues by injecting a large volume of virus with considerable force helps in establishing the infection and is responsible for the almost 100 per cent takes in the control animals. This procedure might well be used when testing dogs for immunity.

Conclusion. A simple test for evaluating the antigenicity of rabies vaccine is described and its advantages and disadvantages are discussed.

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Complementary Correction of the Defective Coagulation Mechanism of Hemophilic and Thrombocytopenic Blood.* (17662)

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Evidence has been presented that at least two factors are necessary for the activation of thromboplastin: one contained in plasma and the other supplied by the platelets. According to Quick(1), active thromboplastin is formed through the action of an enzymatic agent liberated by the platelets (thromboplastinogenase) on a plasmatic precursor (thromboplastinogen). A deficiency of thromboplastin capable of severe impairment of the clotting mechanism may be caused either by lack of thromboplastinogen or of thromboplastinogenase, or by the presence in the circulation of an agent capable of inhibiting the platelet factor (antithromboplastinogenase)(2). All 3 conditions have been found in definite clinical states: the first in hemophilia(1), the second in thrombocytopenic purpura(3,4) and the third in some rare types of acquired hemophilia-like disease(2). Thus in hemophilia there is adequate thromboplastinogenase (platelet factor), but a lack of thromboplastinogen(2). In thrombocytopenic blood there is adequate thromboplastinogen, but a lack of thromboplastinogenase. If these assumptions are correct, the addition of hemophilic to thrombocytopenic blood should produce a mixture with a normal clotting mechanism.

We have recently been afforded the unusual opportunity of studying simultaneously a case of classical hemophilia and a 7-year-old girl with a severe thrombocytopenia (9,700 platelets per cm) of unknown cause presenting, as occasionally found in severe purpura(3), a somewhat prolonged clotting time (23 minutes in glass tubes). Blood from the two patients was mixed in various volumetric proportions. Clotting time, percentage of prothrombin activity left in serum, clot retraction and "accelerator effect" of serum were determined in each sample and in the two original bloods.

Methods. Blood was collected from both

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[*] Aided by grants from the Charlton Fund and the American Cancer Society, Massachusetts Division

[†] This work was completed during the tenure of a Damon Runyon Clinical Research Fellowship, administered by the American Cancer Society.

1. Quick, A. J., *Am. J. Med. Sc.*, 1947, v214, 272.
2. Quick, A. J., and Stefanini, M., Proc. Soc. Exp. Biol. and Med., 1948, v67, 111.
3. Quick, A. J., Shanberge, J. N., and Stefanini, M., *J. Lab. and Clin. Med.*, 1949, v34, 761.
4. Soulier, J. P., *Rév. d'Hématol.*, 1948, v3, 302.

TABLE I.
The Clotting Mechanism of Mixtures of Hemophilic and Thrombocytopenic Blood.

Hemophilic blood, ml	Thrombocytopenic blood, ml	Clotting time, min.	Clot retraction, %	Serum prothrombin activity,* %	"Accelerator effect" of serum, %
2.0	0	42	45	95	6
1.9	0.1	7½	50	80	15
1.8	0.2	6	48	45	18
1.5	0.5	5	44	34	43
1.0	1.0	5	40	25	55
0.5	1.5	5	24	30	37
0	2.0	23	2	90	9

* One hour after completion of coagulation.

patients by venipuncture. A Silicone-coated needle connected with a glass syringe was quickly inserted into a vein and a few ml of blood drawn and discarded. Exchanging this syringe for one coated with Silicone (Dri Film 9987, General Electric Company) and cooled by immersion in an ice bath, 20 ml of blood were withdrawn. The blood was immediately transferred to a Silicone-coated tube kept in an ice bath. Different volumes of hemophilic and thrombocytopenic blood were transferred by means of cooled, Silicone-coated pipettes to Pyrex glass tubes 130 x 10 mm kept in water bath at 37°C. The clotting time of each sample was determined by tilting the tubes every 30 seconds until they could be inverted without spilling the blood. The amount of prothrombin activity left in serum was determined by the one-stage method of Quick(1) according to the technic described by Quick *et al.*(3). The serum prothrombin time obtained was translated into serum prothrombin activity with the use of the dilution curve constructed by the authors(5). The percentage of clot retraction was given as the volume of serum expressed from the clot of 1 ml of whole blood one hour after completion of coagulation (multiplied x 100). The percentage values of "accelerator effect" of serum were determined by the method of de Vries *et al.*(6).

Blood in Silicone tubes from both patients was centrifuged at 800 r.p.m. for 10 minutes to separate the native plasma. The low speed of centrifugation was selected to avoid an excessive loss of platelets by sedimentation. The hemophilic and thrombocytopenic plasmas were then mixed in different proportions. The clotting time, prothrombin activity of serum, clot retraction and "accelerator effect" of serum were determined in each sample, employing the same technics as in whole blood.

Results. Both hemophilic and thrombocytopenic blood gave normal values for fibrinogen, prothrombin and labile factor (factor V, Ac globulin of plasma). Neither contained anticoagulant agents. In both bloods there was evidence of lack of thromboplastin activity as indicated by the high values of prothrombin activity of serum and confirmed by the delayed clotting time and very low "accelerator effect" of serum. When the 2 bloods were mixed in equal volumes, the clotting mechanism became normal (Table I). As the proportion of either blood was increased, the prothrombin activity increased and the "accelerator effect" progressively decreased in serum. However, as little as one part of thrombocytopenic blood in 19 of hemophilic blood reduced the clotting time to normal. Clot retraction, classically defective in thrombocytopenia, became normal when one part of hemophilic was mixed with 3 parts of thrombocytopenic blood. The data presented in Table I are those obtained with mixtures of whole blood; those obtained with native plasma are not given as they were substantially identical.

Discussion. The results presented in this communication support the concept of Brinkhous(7) and Quick(1) that at least 2 factors

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5. Stefanini, M. and Crosby, W. H., to be published.

6. de Vries, A., Alexander, B. and Goldstein, R., *Blood*, 1949, v4, 247.

7. Brinkhous, K. M., PROC. SOC. EXP. BIOL. AND MED., 1947, v66, 117.

are necessary for the activation of thromboplastin: one supplied by the plasma and the other by the platelets. Our results confirm the finding that the platelet factor is quantitatively and qualitatively normal in hemophilia(2) and suggest that the plasma precursor of thromboplastin is normal in thrombocytopenic blood.

A few other data require comment. It will be seen that the addition of 1/20 volume of thrombocytopenic blood to hemophilic blood was sufficient to reduce the clotting time to normal but influenced only slightly the consumption of prothrombin. This indicates that the clotting time of whole blood is less accurate than the prothrombin consumption test as a procedure for the study of hemophilia.

The "accelerator effect" of serum was found to be very low in both hemophilic and thrombocytopenic sera, as previously shown by Alexander and de Vries(8,9). A normal "serum accelerator effect" could, however, be obtained by allowing thrombocytopenic and hemophilic bloods clot in the presence of thromboplastin. In our experiments, the "accelerator effect" of serum appeared to be indirectly proportional to the prothrombin activity of serum.

Summary. In both hemophilic and thrombocytopenic blood the defect of the clotting mechanism reflects a deficiency of active thromboplastin. Addition of thrombocytopenic blood to hemophilic blood results in a mixture in which the clotting time, clot retraction, prothrombin activity and "accelerator effect" of serum all become normal.

These findings support the theory that at least two factors are necessary for the formation of active thromboplastin: a plasmatic agent, deficient in hemophilia, and a platelet factor, defective in thrombocytopenic purpura.

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8. Alexander, B. and de Vries, A., *Blood*, 1949, v4, 747.

9. Alexander, B. and de Vries, A., *Blood*, 1949, v4, 752.

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Tuberculin Reaction. IV. Failure of Pyribenzamine to Protect Against Systemic Tuberculin Shock.* (17663)

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The suppressing effect of antihistaminics on allergic reactions involving histamine or histamine-like substances is well established. Although there is no good evidence to indicate that histamine plays a role in the tuberculin reaction, it naturally becomes of interest to inquire whether antihistaminics will suppress the allergic reaction of tuberculin type sensitivity. Information on this point should be helpful in elucidating the mechanism of tuberculin-type reactions as well as being of possible immediate value in the treatment of diseases in which this type of sensitivity is present. These objectives have led to a limited number of investigations on the effect of various antihistaminics on the course of tuberculosis and cutaneous tuberculin sensitivity in experimental animals and man. The conflicting results of the French investigators have recently been summarized by Wissmer(1) who, in his own experiments, observed that oral administration of 500 mg/day of the powerful antihistaminic R. P. 3015 (dimethylaminoethylthio-diphenylamine) did not suppress cutaneous reactivity to tuberculin in either tuberculous or non-tuberculous patients. Sar-

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1. Wissmer, B., *La Revue d'Immunol.*, 1947, v11, 183.

ber(2) reported that subcutaneous doses of 50 mg/kg/day of Benadryl exert a definite suppression on the cutaneous tuberculin reaction of tuberculous guinea pigs. Friedman and Silverman(3) observed that Pyribenzamine given orally to a large group of tuberculin-positive pre-adolescent children in doses of 60 and 240 mg daily for 4 days did not exert any inhibiting effect on the cutaneous response to tuberculin. Millner and Hurst(4) have reviewed much of the recent literature on the use of antihistaminics in tuberculosis and presented their own observations that prolonged treatment of tuberculous adults with a combination of Neo-antergan and Phenergan in daily dosage of 300 mg and 25 mg respectively did not provide any conclusive evidence that the drugs were beneficial. These authors called attention to the observation emphasized by Rich(5) and many others that the cutaneous tuberculin test is often unreliable because of the lowering of the reactive capacity of the skin by nonspecific factors especially those which affect the circulation. It is apparent that the effects which antihistaminics may exert on the course of tuberculosis and tuberculin sensitivity have not been established.

The present investigation was conducted as part of a project on the mechanism of the tuberculin reaction. The systemic tuberculin test was employed as the measure of tuberculin sensitivity because it is commonly observed to be a more reliable measure of tuberculin sensitivity in experimental animals than the cutaneous test(6).

Experiments. In the first experiment 15 large guinea pigs weighing 600 to 1000 g were given an intraperitoneal dose of 2.8 mg of a young culture of the B.C.G. strain of *M. tuberculosis* var. *bovis.* Twenty-four days later the animals responded to the intracutaneous test with 1:100 Old Tuberculin[†] with strong reactions, 4 of which showed central areas of necrosis. On the 34th day, 11 of the animals were "protected" with a series of subcutaneous injections of Pyribenzamine[‡] in doses expressed per kg body weight and timed with respect to the shock dose of tuberculin as follows: 2 mg 1 hour before shock; 4 mg 20 minutes before shock; 2 mg 1 hour after shock; 2 mg 2 hours after shock; 2 mg 3 hours after shock and finally 2 mg 4 hours after shock to the surviving pigs. All animals were given an intraperitoneal shock dose of 5 ml of 1:5 O. T. There was no difference in the reactions of the two groups and all animals developed typical severe tuberculin shock in from 3 to 4 hours. Of the Pyribenzamine-treated animals, one died during the 4th hour, one during the 5th hour, 2 during the 6th hour, 4 during the 8th hour, and 3 sometime between the 8th and 20th hours. The 4 unprotected controls died during the 4th, 5th, 9th, and 10th hours respectively.

A second experiment was conducted with 18 large guinea pigs similarly infected with B.C.G. They all gave 3+ to 4+ cutaneous reactions to 1:100 deglycerinated O.T. when tested on the 22nd day after infection. Twelve of the animals were given "protective" doses of Pyribenzamine at 2, 1, and 0 hours before the shock dose of 10 ml of 1:5 O.T., and 1 and 3 hours later. The remaining 6 unprotected controls were given the shock dose of tuberculin at the same time. All of these animals developed severe shock between the 2nd and 3rd hour. There was no significant difference in survival time of the treated and untreated animals. Further details on methods and the results of the experiment are given in Table I. Three non-tuberculous controls which received the same Pyribenzamine dosage as the animals of Group II shown in the table

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2. Sarber, R. W., *Am. Rev. Tub.*, 1948, v57, 504.
3. Friedman, E., and Silverman, I., *Am. Rev. Tub.*, 1949, v60, 354.
4. Millner, T., and Hurst, A., *Dis. Chest*, 1949, v16, 870.
5. Rich, A. R., *The Pathogenesis of Tuberculosis*, pp. 368, 369, Charles Thomas, Springfield, Illinois, 1st Edition, 1944.
6. Hehre, E., and Freund, J., *Arch. Path.*, 1939, v27, 289.

[†] The Old Tuberculin used in these experiments was kindly supplied by the Lederle Laboratories of Pearl River, N. Y.

[‡] The Pyribenzamine HCl was kindly supplied by the Ciba Pharmaceutical Products, Inc., Summit, N. J.

TABLE I.
Attempt to Protect Tuberculous Guinea Pigs Against Systemic Tuberculin Shock with Pyribenzamine.

No. of animals	Total dosage Pyribenzamine, mg/kg body wt	Time of death—hr after shock dose of O.T.
	Group I*	
2	5	8 to 20
4	5	4
	Group II†	
1	10	8 to 20
3	10	4
1	10	5
1	8	3
	Group III (controls)	
4	0	4
2	0	5

* Group I pigs received 1 mg/kg at each injection.
† Group II pigs received 2 mg/kg at each injection.

were unaffected by the shock dose of tuberculin.

Additional experiments were conducted on some 50 guinea pigs in which the total dosage of Pyribenzamine was varied from 4 to 12 mg/kg and spaced at intervals between 2½ hours before to 7½ hours after the shock dose of tuberculin. The shock dose of tuberculin was varied from 3.3 to 10 ml of 1:5 O.T. In no instance was there any evidence of protection by Pyribenzamine.

Summary. The results of the various experiments did not provide any evidence that Pyribenzamine protects against systemic tuberculin shock in the tuberculous guinea pig.

This is in contrast to the marked protection Pyribenzamine affords against active anaphylactic shock where as little as 1 mg/kg given subcutaneously 10 minutes before the shock dose of antigen may give full protection against fatal anaphylaxis(7). In our own experience, several properly-spaced doses of Pyribenzamine totalling 4 to 8 mg/kg given subcutaneously at intervals before and after a large shock dose of antigen prevents severe shock and death from both acute and protracted active anaphylaxis in the majority of highly sensitive guinea pigs(8).

The failure of Pyribenzamine to protect against systemic tuberculin shock is perhaps to be expected since no smooth muscle reaction or shock organ has ever been identified as taking part in this type of shock.

The results of the present experiments indicate that histamine or histamine-like substances do not play a significant role in tuberculin shock and support the concept that anaphylactic and tuberculin sensitivities are basically different.

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7. Mayer, R. L., Huttrer, C. P., and Scholz, C. R., *Science*, 1945, v102, 93.

8. Weiser, R. S., unpublished work.

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Histochemical Demonstration of Reducing Activity in Normal and Leukemic Blood and Bone Marrow Cells.* (17664)

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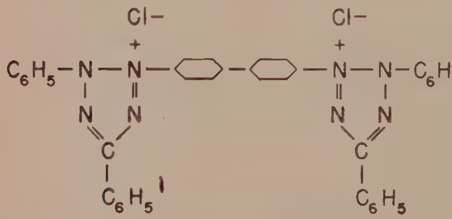
It was found previously that leucocytes from various exudates reduced potassium tellurite and pp′ diphenylene bis 2-(3,5 diphenyl tetrazolium chloride), ("neotetrazolium chloride")(1). The sites of enzymatic activity could be recognized under the microscope either due to the deposition of reduced dark tellurium or the insoluble purple formazan. In the present study, observations were made on normal and abnormal blood and bone marrow cells.

Method: Freshly drawn venous blood was heparinized, thoroughly mixed and centrifuged. The plasma was removed and the upper layer was pipetted into the reagent solution. Aspirated bone marrow or exudate was treated in a similar fashion. Material from lymphnodes was cut into fine pieces in the reagent solution. pp′ diphenylene bis 2-(3,5 diphenyl tetrazolium chloride) was preferred as reagent since purple granules which follow its reduction can be more easily distinguished from preformed refractile granules in blood cells than the dark tellurium deposits. pp′ diphenylene bis 2-(3,5 diphenyl tetrazolium chloride) has the formula:†



The trade name of this substance is "neotetrazolium chloride". It can be obtained from The Pannone Chemical Company, Farmington, Conn. A freshly prepared saturated solution of this substance in M/10 phosphate buffer, pH 7.4 was used. The chemical should be kept in the refrigerator since considerable deterioration was noticed during the hot summer months. The blood or bone marrow suspension was added to the reagent solution at 37°C. A drop of sediment was examined under the microscope after the suspension had been left in the incubator for one hour without further staining. Longer incubation time up to 15 hours did not alter the observed results. The identification of unstained blood cells is admittedly difficult. However, the size and shape of the cell as well as the form of the nucleus and type of cytoplasmic granulation, if present, permits in most cases proper identification. Moreover, by studying leukemic blood in which routine films revealed almost only one type of blood cell, reliable results could be obtained.

Observation. Normal blood: A considerable portion (62-84%) of all white blood cells revealed evidence of reducing activity. Polymorphonuclear leucocytes, lymphocytes and monocytes were seen to contain one to several fine purplish granules. The positive cells were mostly polymorphonuclear leucocytes while only some of the lymphocytes were positive. Eosinophiles contained fine granules which were independent of the coarse eosinophilic granulations.

Leukocytosis. In blood with marked leukocytosis due to various causes, most polymorphonuclear leucocytes contained purplish granules. In some of the cells, these granules were coarser and more numerous than commonly seen in normal leucocytes.

Exudates. Almost all polymorphonuclear leucocytes contained granules that were more numerous and coarser than those seen in normal blood cells (see Fig. 1). Occasionally,

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* This work was supported by a grant from the Damon Runyon Memorial Fund for Cancer Research.

1. Wachstein, M., PROC. SOC. EXP. BIOL. AND MED., 1949, v72, 175.

† The structural formula given in the previous publication(1) shows the chloride ion in the right tetrazine ring erroneously in an asymmetrical position.

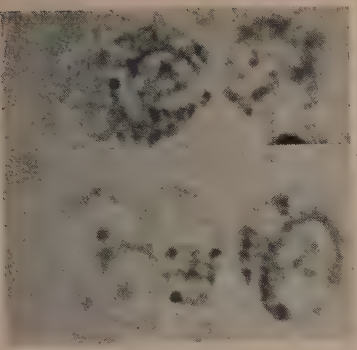

FIG. 1.
Leucocytes from an infected wound showing coarse cytoplasmic granules due to deposition of reduced insoluble pp′ diphenylene bis 2-(3,5 diphenyl tetrazolium chloride). × 1400.

a number of granules were clumped together. In addition, some of the cells contained large vacuoles which stained orange to purplish red.

Acute myeloid leukemia. In the blood of 7 patients with this disease, myeloblasts did not show any staining reaction. Many of the more mature myeloid elements including myelocytes showed purple granules.

Chronic myeloid leukemia. In the blood of 4 patients, a considerable portion of all white blood cells showed reducing activity. As in the acute form, the most immature cells were negative while granules were demonstrable in myelocytes and the more mature forms.

Chronic lymphatic leukemia. Blood from 10 patients with this disease was examined. While in one case none of the lymphocytes were positive, in 5 others the percentage of positive cells varied between 2 and 15. In 3 of the examined cases, 40, 92 and 96% respectively, were positive. In the tenth case, at the first examination the blood contained 137,000 lymphocytes per cu mm of which 19% were positive while three months later it contained 265,000 lymphocytes of which 98% were positive. In the blood of all of these patients, most of the polymorphonuclear leucocytes present showed evidence of reducing activity.

Lymphnodes. Suspensions from tonsils and lymphnodes from surgically removed specimens found to be normal on microscopic examination showed 46-54% of lymphocytes to be positive.

Bone Marrow. In bone marrow suspensions of normal individuals, a considerable portion of all cells revealed reducing activity. The majority of positive cells were found in the myeloid series. Activity could first be identified in myelocytes. Only occasional nucleated red cells showed few purplish granules. Megakaryocytes revealed a varying behavior. In some the cytoplasm was studded with dust-like purple granules while others were entirely negative. In bone marrows from patients suffering from various kinds of anemias, similar findings were obtained. In general only few of the nucleated red cells showed purplish granules. As in the peripheral blood, the myeloblasts in bone marrow suspensions from patients with acute myeloid leukemia were devoid of reducing activity.

Various animals. In the blood of rats, guinea pigs, rabbits and mice, some cells were positive. Apparently the positive cells were of the polymorphonuclear (heterophile) type. In bone marrow suspensions of all of these animals, some of the cells likewise revealed reducing activity.

Comment. Various enzymes are known to occur in white blood cells. In general larger quantities are found in polymorphonuclear leucocytes than in lymphocytes(2). This can be observed under the microscope with several of the enzymes that are demonstrable histochemically *e.g.*, the oxydases and peroxydases and alkaline phosphatase(3). The findings presented here follow the same general pattern since histochemically demonstrable reducing activity is found in normal blood, predominantly in leucocytes. It has been previously reported(1) that in the kidney the deposition of reduced pp′ diphenylene bis 2-(3,5 diphenyl tetrazolium chloride) or tellurium takes place in such a manner as to suggest a localization within the mitochondria. In the blood cells there is, however, apparently an inverse relationship between the occurrence of mitochondria and histochemically demonstrable reducing activity since the mature granulocytes which are almost lacking in these

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2. Barnes, J. M., *Brit. J. Exp. Path.*, 1940, v21, 264.

3. Wachstein, M., *J. Lab. Clin. Med.*, 1946, v31, 1.

cellular constituents show most of it. The behavior of reducing activity in normal and leukemic blood cells is paralleled by the findings as obtained by the Warburg technic. The immature cells have a purely oxidative metabolism without any aerobic lactic acid formation. In contrast, the more mature granulocytes show strong lactic acid formation under aerobic conditions(4). No significant changes are present between granulocytes from normal and leukemic blood(5).

Summary. Reducing activity in blood and bone marrow cells can be demonstrated with the aid of pp′ diphenylene bis 2-(3,5 diphenyl tetrazolium chloride). Under the microscope, sites of reduction are indicated by small purplish granules due to the formation of insoluble formazan. A positive reaction is seen in the myeloid elements with the exception of the myeloblasts. Lymphocytes and monocytes show a varying degree of activity. Nucleated red cells and megakaryocytes are occasionally positive. In leukemic blood, myeloblasts are consistently negative while the more mature elements show reducing activity.

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Significance of the Intestinal Flora in Nutrition of the Guinea Pig.* (17665)

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Considerable work has been done to develop a purified ration which will give growth in guinea pigs equivalent to that obtained with commercial rations. In recent reports (1,2) from this laboratory it was shown that the growth was remarkably improved when gum arabic was added to the purified basal ration. Almost normal growth was obtained when in addition to gum arabic potassium acetate and magnesium oxide were used. The reason for the growth response obtained with gum arabic, potassium acetate, and magnesium oxide could not be explained with certainty. Since it seemed possible that this response was not based on a direct effect on the animal but on a beneficial effect on the intestinal flora, we have investigated the intestinal flora of the guinea pig. The chief purpose was to obtain some indication of the numbers of certain representative organisms and to determine whether there are great differences in the absolute and relative numbers when different diets are fed to the animals. Attention was also paid to the excretion of vitamins from animals on different rations.

Experimental. The procedure for growing the animals was similar to that used by Booth *et al.*(1). Two different rations were used. The *first* one, the synthetic basal ration, consisted of: sucrose 60.9%, casein (vitamin-free) 30%, salts IV[3] 4%, fortified soybean oil 4%, sucrose mixture containing B vitamins 0.8%, and choline 0.3%. The fortified soybean oil supplied 1.2 mg of β-carotene, 12 mg of α-tocopherol, 8 μg of calciferol and 0.2 mg of menadione per 100 grams of ration. The vitamin B mixture supplied 1 mg of thiamine hydrochloride, 1.4 mg riboflavin, 1 mg pyridoxine, 3 mg calcium pantothenate, 200 mg inositol, 10 mg niacin, 10 mg p-aminobenzoic acid, 0.04 mg biotin and 0.3 mg folic

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* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station.

† Rockefeller Foundation Fellow. Present address: Department of Nutritional Chemistry, University of Helsinki, Finland.

1. Booth, A. N., Elvehjem, C. A., and Hart, E. B., *J. Nutrition*, 1949, v37, 263.

2. Roine, P., Booth, A. N., Elvehjem, C. A., and Hart, E. B., PROC. SOC. EXP. BIOL. AND MED., 1949, v71, 90.

3. Hegsted, D. M., Mills, R. C., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, 1941, v138, 459.

TABLE I.
Effect of Different Rations on the Number of Cecal Bacteria in Guinea Pigs.

	Ration	
	Basal	Basal + gum + K-acet. + MgO
No. of animals	16	18
Dry wt. of cecum, g	2.6	3.7
pH of cecal content	7.3	6.8
Aerobic bacteria per g dry cecal content	$6.9 \times 10^6 \pm 2.4 \times 10^6$	$9.9 \times 10^6 \pm 2.5 \times 10^6$
Anerobic bacteria per g dry cecal content	$6.8 \times 10^6 \pm 2.4 \times 10^6$	$28.9 \times 10^6 \pm 9.0 \times 10^6$
Coliform bacteria per g dry cecal content	$7.5 \times 10^4 \pm 4.2 \times 10^4$	$33.6 \times 10^4 \pm 8.4 \times 10^4$
Lactic acid bacteria per g dry cecal content	$4.7 \times 10^9 \pm 1.5 \times 10^9$	$12.6 \times 10^9 \pm 4.7 \times 10^9$

acid per 100 g of ration. Vitamin C dissolved in sucrose solution was fed with individual pipettes at a level of about 25 mg every other day. The *second* ration had the same composition, except that 15% gum arabic, 2.5% potassium acetate, and 0.5% magnesium oxide were added in place of an equivalent amount of sucrose.

As the cecum evidently is the main site of the intestinal flora, all the bacterial work was done on cecal contents. The technic was the same as that used by Nath *et al.*(4). The animals which had been on the experimental rations for 2 to 6 weeks were sacrificed, the ceca removed and the contents squeezed aseptically into sterile Petri dishes. Representative 0.5 g samples were transferred to sterile 50 ml water blanks containing glass beads and shaken thoroughly. From these initial 1:100 dilutions serial decimal dilutions were made up to 10^{-8}. Quantitative inoculation was then made of the respective dilutions into various culture media.

An estimate of the total viable population of aerobic and anaerobic bacteria was made by using the BBL thioglycollate medium. For culturing anaerobes the oat jar method was used and a period of two days at 37°C was allowed for incubation of the plates. Eosin methylene blue agar (Difco) was used for culturing coliform organisms and carrot-liver agar (Foster *et al.*(5)) for the lactic acid bacteria. In preliminary experiments enterococci and yeasts were cultured on special media. Since their numbers were very low and no correlation between them and the rations could be found, these groups of organisms were not counted in the main experiments. The results are summarized in Table I.

It may be seen that the dry weight of the cecal content is somewhat higher in the animals receiving the more complete diet. There is also a difference in the average pH of the cecal content, the pH being slightly alkaline in the animals of the basal group and slightly acidic in the other group. This appears at first somewhat strange, but evidently the final pH is not determined by the pH of the food but by the kind of microflora which has developed in the cecum.

In general the counts of aerobic, anaerobic, and coliform organisms per gram of cecal dry matter are very low, much lower than the values of Nath *et al.*(4) for rats on sucrose diets. Only the numbers of lactic acid bacteria are high, even higher than those of the rats; lactic acid bacteria seem to be predominant organisms in the cecum of guinea pigs.

The individual variations in the bacterial counts of different animals were very high. To be able to draw any conclusions regarding the differences in the two groups of animals statistical treatment of the results was used. The standard errors of the means are given in the table. In addition a "t-test" was made according to Fisher(6) to determine whether

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4. Nath, H., Barki, V. H., Sarles, W. B., and Elvehjem, C. A., *J. Bact.*, 1948, v56, 783.

5. Foster, E. M., Garey, J. C., and Frazier, W. C., *J. Dairy Sci.*, 1942, v25, 323.

6. Fisher, R. A., *Statistical Methods for Research Workers*, Edinburgh, 1938, 7th Ed.

the differences found between the groups of animals were statistically significant. The P values were found to be between .3 and .4 in the group of aerobes, between .02 and .05 in anaerobes. between .01 and .02 in coliforms and between .1 and .2 "lactics." These values show that there are significant differences in the groups of anaerobic and coliform bacteria. In the groups of aerobic and lactic acid bacteria, on the other hand, the differences are not significant.

A more detailed picture concerning relationships between the ration and the intestinal flora of the animals can be obtained only by new extensive studies with large numbers of animals. According to the results obtained, it is evident that there are significant differences in the composition of the intestinal flora between the animals on the basal diet and those on the gum arabic, potassium acetate, and magnesium oxide containing diet, *i.e.*, between slower and faster growing animals. These differences can manifest themselves, *e.g.*, in an increased intestinal synthesis of vitamins or, on the other hand, in an increased destruction of vitamins, or possibly even in an increased synthesis or destruction of toxic substances.

An experiment was made to determine whether there are differences in the vitamin excretion from guinea pigs on the different diets. The vitamins investigated were riboflavin and folic acid. Six animals were put on the basal diet and six on the basal + gum arabic + potassium acetate + magnesium oxide diet. After 3 weeks on such a dietary regimen the animals were placed in metabolism cages for 2 days and the urine was collected. During this collection period the diets did not contain any riboflavin or folic acid. The amounts of riboflavin and folic acid in the urine were determined microbiologically. The results are given in Table II.

TABLE II.
Effect of Different Rations on Urinary Excretion of Riboflavin and Folic Acid.

	Ration	
	Basal	Basal + gum + K-acet. + MgO
No. of animals	6	6
Riboflavin excreted, μg/day	11.9	19.8
Folic acid excreted, μg/day	0.13	0.76

As can be seen there are differences in the excretion of both of the vitamins studied. Particularly the difference in folic acid excretion is very distinct. If the amounts excreted are compared with the levels ordinarily fed to the animals, it is evident that they are rather low. In spite of this they point to important differences in the vitamin economy of the animals on different diets. These differences might be in the synthesis, in the extent of retention, or in the rate of destruction of vitamins.

Summary. An investigation was made on the cecal flora of guinea pigs receiving a purified basal diet and the basal diet supplied with gum arabic, potassium acetate and magnesium oxide. Significantly more coliform and anaerobic bacteria were found in the ceca of animals on the more complete diet. The urinary excretion of riboflavin and folic acid was also greater in this group of animals. It seems probable that the beneficial effect of gum arabic as well as that of potassium acetate and magnesium oxide on the growth rate of guinea pigs is based on the formation and maintenance of a proper intestinal flora. Evidently there is a well balanced symbiosis between the host animal and the different bacteria, and the bacterial flora has important functions in the physiology of the animal. Artificial, purified diets can easily disturb this balance and thus produce nutritional disturbances in the animal. On a basal purified diet growth is always very poor. The gum arabic induces conditions in which a proper flora can develop. For a normal flora and normal growth other supplements like potassium and magnesium salts are usually needed.

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Effect of *Lithospermum ruderale* on Oestrous Cycle of the Rat.[*] (17666)

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In 1941 a treatise by the United States Department of Agriculture on the medicinal uses of plants by Indian tribes of Nevada(1), contained the following annotation on *Lithospermum ruderale.* "In one settlement, it is believed that the plant has contraceptive properties, and that a cold water infusion of the roots, taken daily for a period of 6 months, will induce sterility thereafter." Alcohol extracts of lithospermum administered to mice, in their diet, inhibit or abolish the normal oestrous cycle and reduce the incidence of birth.(2-4) Further work has shown that the gonadotrophic content of pituitaries of mice on a lithosperm diet was reduced, and the conclusion was drawn that the primary action of the drug was a depression of pituitary activity(5,6). However, more extensive experiments have not supported this conclusion(7,8). When work on lithosperm began in our laboratory 2 years ago, the roots and remainder of the plant (tops) were dried and ground separately. Experiments were then carried out as follows.

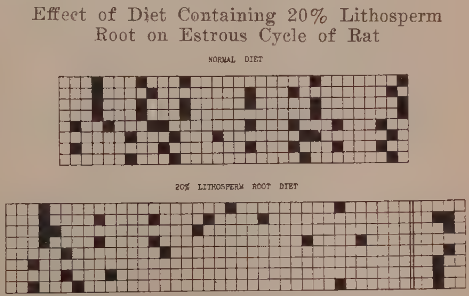

Fig. 1
Double line indicates return to normal diet; black squares represent vaginal cornification.

Oral administration to rats. Eight adult Sprague Dawley female rats were placed on an *ad lib.* diet of Purina fox chow for 36 days. Following this the diet was changed to one consisting of 20% dry lithosperm roots and 80% Purina chow, for an additional 36 days. The oestrous cycle of these animals was followed throughout by daily vaginal smears and the results of this experiment may be seen in Fig. 1. The experiment was then repeated using lithosperm tops, in concentrations of 20% and 25% of the diet. However, the effect of tops on the oestrous cycle was negligible.

About one year later part of the experiment was repeated, using a water extract of the original dried root. This extract was prepared by extracting twice with cold water, and finally with water at 90°C. An amount of extract equivalent to 200 mg of dried root, was fed daily by stomach tube for 10 days to 5 adult female rats, and no effect was noted on the oestrous cycles. The dose was then doubled to an equivalent of 400 mg/day, and a questionable inhibition of oestrous cycles was obtained.

Other feeding experiments have been carried out, using a lyophilized water extract of lithosperm, kindly supplied by Ayerst, Mc-

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[*] This work was supported by a grant from the Ontario Cancer Treatment and Research Foundation.

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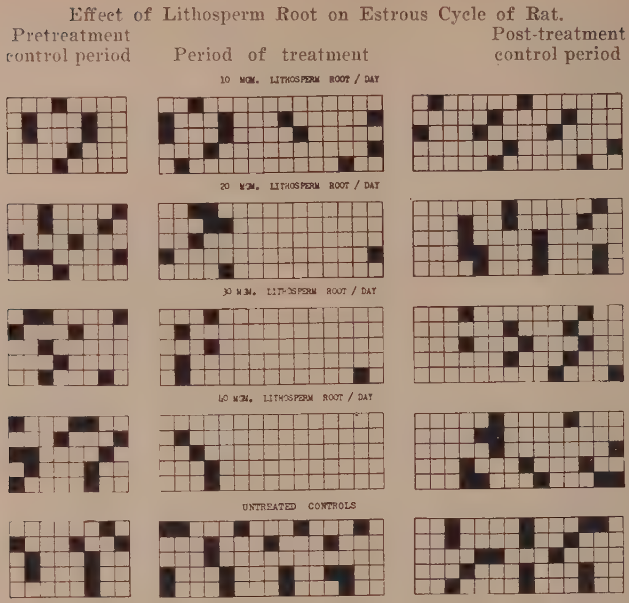

Fig. 2

Kenna and Harrison of Montreal. This extract again was roots and tops, and represented about a ten-fold concentration of the crude root, and a 3-fold concentration of the tops of the plant. Daily tube feeding of 400 mg of lyophilized root extract did not appear to produce any inhibition of the oestrous cycles, nor did doubling this amount have any effect. In various feeding experiments some reduction in weight gain may be noted. This is usually transitory and of a small magnitude. In control experiments considerably greater weight loss may be induced without interruption of the oestrous cycle.

Subcutaneous injection of extracts. Extensive work has now been done which indicates that the effects of subcutaneous injection of lithosperm extracts are much greater than those following oral administration. A typical experiment is as follows.

Daily vaginal smears were done on 5 groups of 5 Sprague Dawley female rats for 37 days. During the first 8 days no treatment was given. For the 2nd period of 15 days, the first 4 groups of rats received 10 mg, 20 mg, 30 mg and 40 mg respectively of lyophilized extract of lithosperm roots. The 5th groups received no treatment. During the remaining period of 14 days no injections were given. It may be seen in Fig. 2 that 10 mg had only slight effect on the oestrous cycle, but that the larger doses produced almost complete anoestrous, this effect appearing after 3 or 4 days. Extracts of tops, in comparable experiments, though somewhat less active, showed the same inhibitory effect.

Effect of Oestradiol on Lithosperm Treated Rats. Sixteen bilaterally ovariectomized adults were injected daily for 3 days with a total dose of 1 μg of oestradiol. Eight of these rats were injected subcutaneously daily with 80 mg of lithosperm root, starting the day prior to oestrogen and continuing throughout the experiment. Daily vaginal smears showed that 7 of the 8 lithosperm treated animals came into oestrous, while all the controls showed vaginal cornification. This experiment supports Cranston's original observation, that lithosperm does not inhibit the action of oestrogen.

Oestrogenic Activity of Lithosperm. The presence of a water soluble oestrogen has been noted during the course of various experiments with lithosperm extracts. It is more abundant in the tops than the roots, but both preparations may be shown to be oestrogenic by direct application to the vaginas of ovariectomized rats. The oestrogen content varied from one extract to another, but 200 mg of lyophilized tops may contain up to 1 γ equivalent of stilboestrol, when assayed on the uterine weights of immature rats. The oestrogen content of lithosperm is a factor which may influence the gonadotrophic content of the pituitaries of treated animals and throws some doubt on the interpretation of previously published assays.

Summary. It has been shown that *Lithospermum ruderale* has an anti-oestrous effect on rats, the roots containing more active material than the tops. The subcutaneous injection of extracts of the plant is at least 10 times more effective than oral administration. Lithosperm does not inhibit the action of administered oestradiol. Experiments indicate that lithosperm contains a water soluble oestrogen which is found primarily in the tops.

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Value of Daphnia as an Assay Organism for Veratrum Alkaloids. (17667)

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Veratrum plants contain an unknown number of alkaloids of apparently similar but not identical physiological action, which are not amenable to chemical assay. The availability of a biological testing method would, therefore, be of decided advantage. Recently, *Daphnia magna* has been extensively publicized as a testing object. Daphnia have been recommended before for the assay of cardiotonic drugs(1). Concerning the adaptation of the test for Veratrum, the only information available is the statement that the criterion of potency consists in the arrest of the heart of *Daphnia magna*. It is evident that this possibly measures a toxic effect not necessarily proportional to the therapeutic value of the material. Another point is that any analogy between Daphnia and human being cannot be postulated. For the present study we prepared jervine, veratrosine, pseudojervine and veratramine following the methods of Jacobs and Craig(2). Having difficulty in obtaining *Daphnia magna*, our first experiments were performed with a non-identified Daphnia strain obtained from a New Jersey pond; later we repeated the work with *Daphnia magna*.

To make these tests, a capillary tube, bent slightly downward at the end, is fixed with Scotch tape to an object carrier with a ground hollow in such a way that the tip rests at the edge of the hollow. A small piece of absorbent cotton is placed into the hollow without touching the capillary. The latter is connected to a suction pump. Daphnias are caught with a wide-tipped medicinal dropper and placed in the center of the cotton bed. The excess of water is promptly removed by the suction but sufficient liquid remains in the cotton to cover the animals caught between the fibers, provided the capillary does not touch the cotton. It is feasible to observe 4 or 5 small Daphnias at a time but only one or 2 of the *magna* species. The medication, in proper dilution, is then dropped from a pipette on the cotton until the water is replaced by the solution. From this moment on the time of action is counted. Every 3 to 4 minutes one-half cc of the solution is flown through the cotton to prevent a change in concentration by evaporation. The normal heart rate of the Daphnia ranges from 250 to 600 beats per minute(1), which is beyond the possibility of counting. Under the influence of the alkaloids the rate

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1. Boylor, E. R., *Biol. Bull.*, 1942, v83, 165.

2. Jacobs, W. A., and Craig, L. C., *J. Biol. Chem.*, 1944, v155, 565; 1945, v160, 555.

TABLE I.
Response of Wild Daphnia to Veratramine at Varying Concentrations.

Veratramine conc.	pH	Total No.	Heart failure within						Remarks
			6 min.	12 min.	20 min.	21-30 min.	31-40 min.	40 min. +	
1:1000	5	13			2	1	2	8	Adults
1:1000	3	6	3	3					’’
1:1000	4	57	0	7	31	11	7	1	’’ most bradycardia
		23	0	5	13	5			Young D. most dead
1:1330	4	35	0	3	12	8	6	6	Adults
		9	0	1	6	1	1		Young all dead
1:2000	4	6				4	2	2	Adults
1:3000	4	20					6	14	
1:5000	4	24					4	20	
1:50,000	4	9						9	

Neither veratrosine 1:500, jervine 1:500 nor pseudojervine 1:2000 (max. possible conc.) produced more than a moderate bradycardia within the time of observation, 40 to 60 min.

soon drops to 180 or less which permits counting with the aid of a stop watch. It was soon found that even the same alkaloid does not have a uniform effect on the Daphnia heart. Some hearts slow down gradually until they finally stop, others keep up a slow beat for an indefinite time; some come to a stop from a relatively fast beat, which stop may be final or followed by periods of resumed beating. Some hearts develop arrhythmias. All these signs may be considered as symptoms of heart failure.

It was necessary to select somewhat arbitrarily the following criteria as end point; stop of the heart, regardless whether temporary or final; pronounced arrhythmias; drop of the heart rate to 75 or less. Another effect was observed in some rare instances: a gradual decrease in amplitude at sustained fast rate until finally only a vibration of the heart membrane remained. Since an endpoint in this case would be absolutely arbitrary, the few animals giving this reaction were not included in the compilation. *Daphnia magna* showed to some alkaloids reactions not seen in the wild Daphnia: a quick stop of the heart within a few minutes followed by resumed beating lasting some minutes and alternating stops and activity. In response to weak solution this behavior may last for more than an hour. A limitation was set to the tests by the low solubility of the alkaloids. A solution in dilute hydrochloric acid, neutralized to pH 5, remains clear, but the alkaloids have little effect in such solutions. The activity was manifest at higher acidity. Since Daphnias showed no deterioration in hydrochloric acid of pH 4 during one hour, all solutions were adjusted to that pH.

Table I shows the effect of veratramine on wild Daphnias. The other alkaloids tested at highest possible concentrations produced a moderate degree of bradycardia within 40 minutes but the signs of heart failure did not appear at all or only after a long and variable period of time. Table II shows the results obtained with *Daphnia magna*. The striking difference is the high potency of veratramine if the quick and temporary arrest of the heart is taken as an endpoint. It was obtained at dilutions about 100 times higher than those effective in the wild strain. The sudden, temporary stop seems to be produced by a different mechanism than the slow-developing bradycardia. It fails to appear if the concentration of the solution is increased gradually from subthreshold concentration to the supposedly active strength. Jervine, applied gradually in increasing dosage or at 1:2000 ten minutes preceding veratramine 1:2000, prevented the sudden arrest by the latter. Instead, bradycardia developed progressively to reach the limit of failure after 30 to 40 minutes. Jervine applied simultaneously with veratramine did not prevent sudden heart stop. That the time necessary to produce a certain degree of bradycardia is subject to wide variations may be judged from

TABLE II.
Response of Adult *Daphnia magna* to Four Veratrum alkaloids at pH 4.

Alkaloid and conc.	Total No.	Temporary heart stop		Heart failure in				Remarks
		6 min.	12 min.	20 min.	21-30 min.	31-40 min.	40 min. +	
Pseudojervine								
1:2000	13			1		4	8	
Jervine								
1:10,000	1						1	Bradycardia only; even those with temp. stop recover; none dead at 60′
1: 2,000	11				5	2	4	
1: 1,330	1						1	
1: 1,000	10				2	3	5	
1: 500	10	6	2		2			
Veratramine								
1: 10,000	1	1						
1: 50,000	12	10			2			
1:100,000	12	10		1	1			
1:150,000	10	7		1	2			
1:200,000	16	10	2	1			3	
1:400,000	12				2		10	
Veratrosine								
1:100,000	1						1	
1: 50,000	1						1	
1: 10,000	1						1	
1: 1,000	5						5	
1: 500	24	1			1	8	14	Moderate bradycardia

the following figures obtained with veratrosine 1:500, which alkaloid gives the most dependable responses: The heart rate of 5 Daphnias was determined every 3 to 4 minutes. A rate of 150 was observed at 4 to 8 minutes, 124 at 7 to 32 minutes, 100 at 9½ to 40 and 75 at 27 to 50 minutes.

Veratrum contains a number of other alkaloids not included in these tests. The present investigation leads to the conclusion that the effect of a crude extract, measured by the sudden heart stop, would essentially be caused by veratramine-type alkaloids. This sudden stop makes it impossible to use the bradycardia, that develops slowly, as a criterion for the assay of mixtures.

Krayer(3) has concluded from experiments in mammals that veratramine and veratrosine are of a potency to abolish adrenaline tachycardia, which is within the same range, the latter alkaloid being more potent and less toxic. This type of effectiveness of veratrosine is not likely to be noticed in the Daphnia test.

Conclusions. The Daphnia test actually measures toxicity. The assay of a crude extract would measure the presence of veratramine and of substances of similar action but would not reveal anything about the therapeutic value, if such should exist.

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3. Krayer, Otto, *J. Pharm. and Exp. Therap.*, 1949, v97, 264.

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